THE UNIVERSITY OF ALBERTA

THE TRANSDUCTION OF INFLAMMATORY STIMULI
IN HUMAN NEUTROPHILS

BY

© COLETTE F. STRNAD

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The transduction of inflammatory stimuli in human neutrophils" submitted by Colette F. Strnad in partial fulfilment of the requirements of the degree of Doctor of Philosophy in Pharmacology.

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ABSTRACT

In an effort to learn more about the transduction of chemotactic stimuli in human neutrophils, the effects of various pharmacological probes on the biochemical and physiological events associated with the inflammatory response were explored. The calcium ionophore, A23187, and the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), were both found to elicit superoxide production and lysozyme release in the neutrophil. The responses to sub-optimal concentrations of PMA and A23187 were synergistically enhanced when the two stimuli were administered in combination. This synergism appeared to be based on their ability to mimic selectively the protein kinase C and calcium mobilization limbs of the bifurcating phosphoinositide-dependent transduction process. Interactive effects were also observed between protein kinase C and the adenylate cyclase pathway. While PMA did not alter cyclic AMP levels in resting neutrophils, it did enhance cyclic AMP production in response to prostaglandin E_1 and the chemoattractant, formylmethionyl-leucyl-phenylalanine (fmet-leu-phe), effects which may be ascribed to the suppression of a degree of guanine nucleotide regulatory protein- (G-protein-) imposed constraint on agonist-stimulated adenylate cyclase activity.

Introduction of non-hydrolyzable guanine nucleotide analogues into the cytosol of saponin-permeabilized cells was found to result in an enhanced lysosomal enzyme release response upon the subsequent addition of calcium ions. The site of action of guanine nucleotide-induced

degranulation was proposed to be a G-protein (N_n) interposed between chemoattractant receptors and the phosphoinositide-specific phosphodiesterase. As fluoride ion, a known activator of both the stimulatory and inhibitory guanine nucleotide regulatory proteins of the adenylate cyclase system (N_s and N_i), is an effective activator of superoxide production in the neutrophil, efforts were made to determine whether these effects were occurring at the level of N_n. Fluoride activation of the respiratory burst was a calcium-dependent process, characterized by a prolonged lag period and extended duration of action which were inconsistent with a receptor-initiated mechanism of action, but consistent with the persistent activation of an integral membrane protein. Fluoride activation was found to be associated with an increase in the cellular content of inositol phosphates, the water-soluble products of phosphoinositide turnover. Inositol phosphate accumulation was paralleled by a sustained elevation of cytosolic Ca^{2+} concentrations, as well as by the redistribution of protein kinase C from the cytosolic to the membrane compartment. In contrast to the fmet-leu-phe-evoked superoxide production, Ca^{2+} mobilization, and phosphoinositide turnover responses, those evoked by fluoride were resistant to pertussis toxin inhibition, suggesting that fluoride serves to activate N_n by a GTP-independent process which is unaffected by ADP-ribosylation of the G-protein.

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
AU	absorbance units
cAMP	adenosine 3',5'-cyclic monophosphate
cyt. c	cytochrome c
dpm	disintegrations per minute
fmet-leu-phe/FMLP	formylmethionyl-leucyl-phenylalanine
GDP	guanosine 5'-diphosphate
GMP	guanosine 5'-monophosphate
Gpp(NH)p	5'-guanylylimidodiphosphate
GTP	guanosine 5'-triphosphate
GTPγS	guanosine 5'-3-thio-triphosphate
HBSS	Hanks' balanced salt solution
HEPES	N-2-hydroxyethyl-piperazine-N'-2'-ethanesuphonic acid
$InsP_1$	inositol phosphate
$Ins(1)P_1$	inositol(1)phosphate
$Ins(4)P_1$	inositol(4)phosphate
$InsP_2$	inositol bisphosphate
$Ins(1,4)P_2$	inositol(1,4)bisphosphate
$InsP_3$	inositol trisphosphate

$Ins(1,3,4)P_3$	inositol(1,3,4)trisphosphate
$Ins(1,4,5)P_3$	inositol(1,4,5)trisphosphate
$Ins(1,3,4,5)P_4$	inositol(1,3,4,5)tetrakisphosphate
K/kD	kilodalton
K_D	dissociation constant
LTB_4	leukotriene B_4
min	minutes
mL	millilitre
mM	millimolar
µM	micromolar
NAD	nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
N_i	inhibitory G-protein of adenylate cyclase system
nM	nanomolar
N_n	stimulatory G-protein of neutrophil transduction
N_s	stimulatory G-protein of adenylate cyclase system
n.s.	not significant
O_2^-	superoxide radical
p	probability
PGE_1	prostaglandin E_1
PMA	phorbol 12-myristate 13-acetate
pmol	picomole

PtdIns	phosphatidylinositol
PtdIns(4)P_1	phosphatidylinositol-4-monophosphate
PtdIns(4,5)P_2	phosphatidylinositol-4,5-bisphosphate
Quin 2/AM	quin 2 acetoxymethyl ester
ScAMP-TME	succinyl tyrosine methyl ester derivative of cAMP
sec	seconds
SEM	standard error of the mean
T	transducin
TCA	trichloroacetic acid
X g	times gravity

CHAPTER 1

INTRODUCTION

I. THE TRANSDUCTION OF INFLAMMATORY STIMULI IN HUMAN NEUTROPHILS

A. THE NEUTROPHIL

The neutrophil is a terminally differentiated phagocytic leukocyte which, upon release from the bone marrow, circulates in the vascular system where it normally constitutes 93 to 96 percent of the granulocytic component of human blood. Its physiological role is defense of the host organism against foreign material. Upon activation by appropriate stimuli, such as bacterial products or complement factors, neutrophils penetrate the endothelial barrier of the post-capillary venules to accumulate at the site of infection in the target tissue, a process called chemotaxis. The neutrophil is equipped with a number of cellular functions instrumental in the eradication of offensive material. Objects recognized as foreign are phagocytosed into membrane enclosed vacuoles which in turn fuse with intracellular granules containing hydrolytic enzymes and antibacterial factors. The human neutrophil contains two major classes of granules: the azurophilic and the specific. Whereas the azurophilic granules contain myeloperoxidase, acid hydrolases such as β-glucuronidase, and a limited supply of lysozyme, the specific granules are rich in lysozyme, as well as containing lactoferrin and a vitamin B-binding protein (review Gallin, 1984). A third class of small gelatinase-containing granules has also been described (Dewald et al., 1982). It is speculated that this granular pool may be fated predominantly for fusion with the plasma membrane, as opposed to phagocytic vesicles.

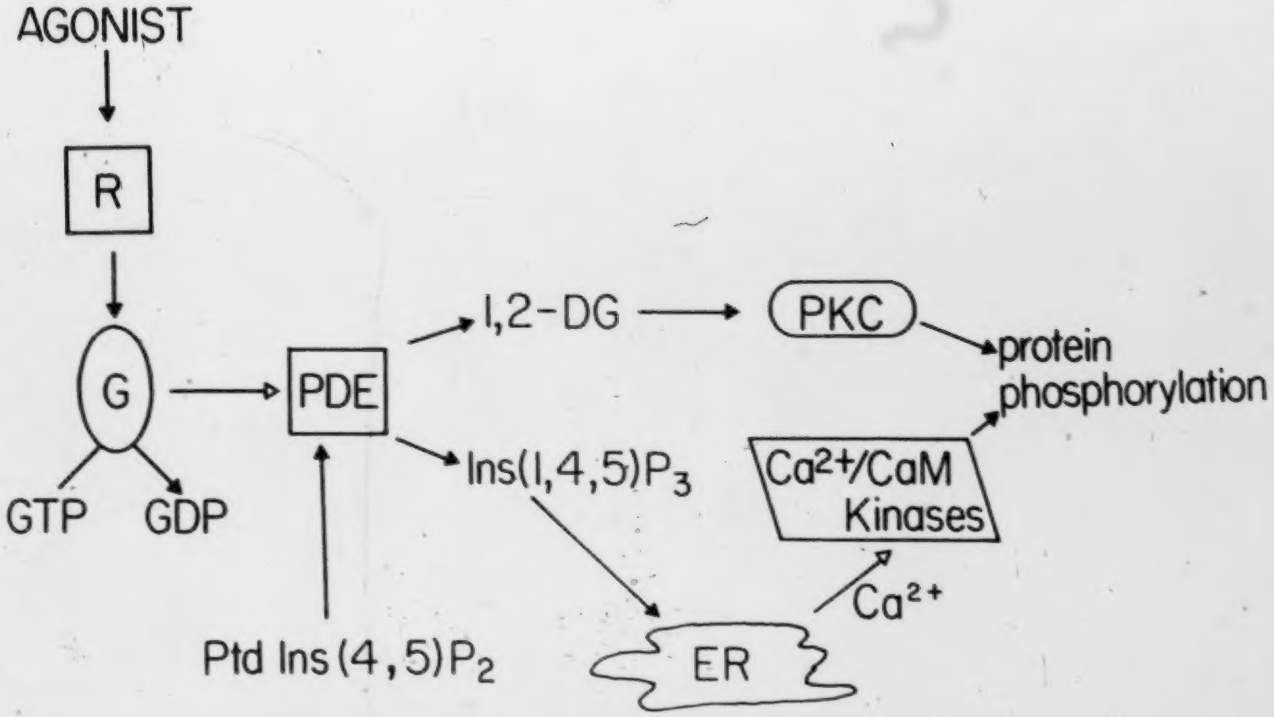
The plasma membrane is equipped with NADPH oxidase, an O_2 reductase, which, in resting cells, exists in a latent state (Babior et al., 1976; Patriarca et al., 1971). Upon activation, this enzyme complex generates superoxide radicals, a phenomenon known as the respiratory burst (Tauber, 1982). NADPH oxidase is a flavoenzyme which accepts a pair of electrons from NADPH in the course of a hydride transfer reaction which results in the reduction of two oxygen molecules (Babior et al., 1976).

$$NADPH + 2O_2 \longrightarrow NADP^+ + H^+ + 2O_2^-$$

The exact sequence of events in the electron transport process remains unclear. Cell activation may be associated with the translocation of certain components of the electron transport chain from intracellular organelles to the plasma membrane (Borreagaard et al., 1984; Ohno et al., 1985). Superoxide, in concert with its reactive derivatives, hydrogen peroxide, the hydroxyl radical, and other products of the myeloperoxidase-H_2O_2-halide system, has toxic effects on microorganisms and neoplastic tissue (Johnson et al., 1975). As foreign material is phagocytosed by the neutrophil, oxidase complexes located in the plasma membrane become part of the phagocytic vesicle. The release of free radical species within the phagolysosome contributes to the destruction of the engulfed material (Cohen et al., 1980). However, the concomitant release of granular enzymes and free radicals into the extracellular space may contribute to inflammatory lesions in the host organism, in addition to the degradation of foreign and transformed material.

Under most physiological and pathological circumstances, neutrophil activation is achieved at the level of cell surface receptors bearing recognition sites for chemotactic factors such as immunoglobulins, complement fragments, and formylated bacterial peptides. In vitro, a commonly applied stimulus is the synthetic tripeptide, formylmethionyl-leucyl-phenylalanine (fmet-leu-phe). Interaction of chemotactic substances with appropriate recognition sites on the receptor molecules induces conformational changes in the latter which influence their relationship with certain integral membrane proteins, characterized by the ability to bind guanine nucleotides. These guanine nucleotide-binding proteins (G-proteins) communicate with a phosphoinositide-specific phosphodiesterase. In its activated form, the phosphodiesterase cleaves the polyphosphoinositide, phosphatidylinositol 4,5-bisphosphate, so generating two second messenger substances: inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate is a water-soluble product which results in the mobilization of calcium from intracellular stores, a response which appears to lead to the activation of various calcium-/calmodulin-dependent protein kinases. The lipophilic product, 1,2-diacylglycerol, is a substance believed to be the physiological activator of protein kinase C. A schematic representation of this pathway is illustrated in Figure 1. The various stages of the transduction process will be dealt with in more detail in the following sections.

Figure 1. Schematic representation of the major molecular events involved in neutrophil activation (R = receptor, G = G-protein, GTP = guanosine triphosphate, GDP = guanosine diphosphate, PtdIns(4,5)P_2 = phosphatidylinositol 4,5-bisphosphate, PDE = phosphodiesterase (phospholipase C), 1,2-DG = 1,2-diacylglycerol, Ins(1,4,5)P_3 = inositol 1,4,5-trisphosphate, ER = endoplasmic reticulum, Ca^{2+} = calcium ion, PKC = protein kinase C, Ca^{2+}/CaM Kinases = calcium-/calmodulin-dependent kinases).


AGONIST
R
G
GTP
GDP
PDE
Ptd Ins(4,5)P$_2$
1,2-DG
PKC
Ins(1,4,5)P$_3$
ER
Ca^{2+}
Ca^{2+}/CaM Kinases
protein phosphorylation

RECEPTORS FOR CHEMOTACTIC FACTORS

The fmet-leu-phe receptor is an extensively glycosylated protein, having an apparent molecular weight in the range of 55K to 70K, as estimated by the migration of labelled receptors on sodium dodecyl sulfate-polyacrylamine gel electrophoresis (Neidel et al., 1980; Painter et al., 1982). Sedimentation equilibrium analysis indicates that the receptor exists as a molecular mass of 63K (Allen et al., 1985). Enzymatic removal of the carbohydrate residues leaves a 32K protein which retains the capacity for ligand binding (Malech et al., 1985).

Approximately 55,000 binding sites are present per cell (Koo et al., 1982). Computer analysis of binding data by non-linear least squares curve fitting is consistent with the co-existence of two classes of receptors exhibiting affinity constants (K_D) in the order of 0.5 and 24 nM, with the high affinity state representing about 25% of the total receptor population (Koo et al., 1982). Similar binding parameters were reported by Mackin et al. (1982). The identical dissociation kinetics observed with chemical and isotopic dilution techniques, suggest the presence of two subpopulations of receptors rather than a negative cooperativity phenomenon. The ability of a series of synthetic formylated peptides to compete with fmet-leu-[^{3}H]phe for receptor binding, with a potency parallelling their efficacy as inflammatory stimuli, indicates a common receptor for these chemoattractants (Williams et al., 1977). Although, the properties of formylated peptide binding are perhaps best characterized, it is important to note that specific binding sites have likewise been identified for other chemoattractants, such as the complement fragment, C5a, and leukotriene B_4 (Snyderman and Pike, 1984).

A submaximal chemoattractant-induced exocytosis of the specific granules has been observed to lead to an increase in the number of fmet-leu-[^{3}H]phe binding sites in intact cells (Gallin et al., 1978; Fletcher and Gallin, 1980; Fletcher et al., 1982). As binding activity is highest in subcellular fractions representative of the specific granules (Fletcher and Gallin, 1983), it is postulated that neutrophils contain an intracellular pool of fmet-leu-phe receptors which are recruited to the plasma membrane in the course of chemoattractant-induced degranulation.

Following ligand binding, much of the receptor protein becomes nondissociable from the plasma membrane and is subsequently down-regulated by means of an endocytotic process, whereupon it is detected in association with the Golgi apparatus (Sklar et al., 1984). Membrane preparations obtained from cells stimulated with formylated peptides contain receptors which are complexed in a high-affinity (slowly-dissociating), high-molecular weight form which, in Triton X-100 extracted cells, co-isolates with the insoluble pellet representative of the cytoskeletal material (Jesaitis et al., 1984). Similarly, radiolabelled leukotriene B_4, upon addition to neutrophils, has been found to form rapid associations with the cytoskeleton (Naccache et al., 1984). Notably, actin polymerization has been used as a measure of neutrophil activation (Roa and Varani, 1982; White et al., 1983). The induction of receptor-cytoskeletal interactions may be instrumental in the regulation of ligand-receptor endocytosis, as indicated by the ability of dihydrocytochalasin B to inhibit the formation of the high affinity fmet-leu-phe-receptor-cytoskeleton complex, as well as its subsequent receptor-mediated endocytosis (Jesaitis et al., 1985). Cytochalasin B is a fungal alkaloid which binds to the growing ends of actin filaments and to actin attachment sites on plasma membrane proteins (Lin et al., 1983). However, although the enhancement of degranulation, superoxide production, and aggregation in cytochalasin B-treated cells is generally thought to be related to its influence on the cytoskeleton, the precise mechanism of this effect remains to be elucidated.

THE GUANINE NUCLEOTIDE-BINDING PROTEIN

The first indications of a guanine nucleotide-dependent regulatory event in a cellular transduction pathway were observed in the glucagon-sensitive adenylate cyclase system of rat hepatocyte plasma membranes. In this system GTP was found to decrease the affinity of glucagon-binding sites (Rodbell et al., 1971a), as well as to enhance the initial rate of both basal and glucagon-stimulated adenylate cyclase activity (Rodbell et al., 1971b). Subsequent studies (Londos et al., 1974; Schramm and Rodbell, 1975) demonstrated that 5'-guanylylimidodiphosphate (Gpp(NH)p), a non-hydrolyzable analogue of GTP, both potentiated the effects of hormonal agents that stimulate adenylate cyclase and activated the cyclase in the absence of hormonal stimulation. Notably Gpp(NH)p, promoted a persistently activated state of adenylate cyclase, in contrast to the readily reversible state of activation achieved with GTP. These findings led to the speculation that GTPase activity might be responsible for the transient duration of GTP effects on the cyclase.

Using affinity chromatography, Pfeuffer (1977) partially purified the G-regulatory unit from detergent extracts of pigeon erythrocyte membranes which had been labelled and pre-activated with photoreactive GTP derivatives. Notably, adenylate cyclase activity was lost upon passage of the extract through a GTP matrix, where the complex dissociated into two protein components, with the GTP-binding unit being retained on the column. Reactivation of the adenylate cyclase activity could be achieved upon eluting the G-protein with GTP or Gpp(NH)p, then recombining it with the unadsorbed fraction.

Additional information concerning the nature of this GTP-binding protein was obtained from studies using mutant strains of the S49 mouse lymphoma cell line. The cytocidal effects of cAMP in wild-type S49 cells provide a criterion for the selection of genetic variants with molecular aberrations in the adenylate cyclase transduction system (Bourne et al., 1975a). One mutant, cyc^-, possesses a normal complement of β-adrenergic receptors and a Mn^{2+}-stimulatable adenylate cyclase activity, but is unable to produce cAMP in response to β-adrenergic agonists, fluoride, non-hydrolyzable guanine nucleotides, or cholera toxin, a bacterial toxin which results in persistent activation of the adenylate cyclase system through an ADP-ribosylation reaction (Bourne et al., 1975b; Insel et al., 1976). However, reconstitution of cyc^- membranes with detergent extracts from the plasma membranes of adenylate cyclase-competent, but β-adrenergic receptor-deficient, cells conferred upon the cyc^- cyclase system the ability to respond to hormonal stimuli and guanine nucleotides (Ross and Gilman, 1977a). Notably, such a reconstitution was still possible when the cyclase activity in the donor extract was destroyed by means of heat inactivation or treatment with sulfhydryl reagents (Ross and Gilman, 1977b; Ross et al., 1978). These studies led to the proposal that cyc$^-$ membranes lacked a transducing component which was necessary both for communication between the receptor and the cyclase catalyst and for activation by guanine nucleotides, fluoride, and cholera toxin. The concept of a G-protein involved in cyclase activation (alternatively designated N_s or G_s) was thus founded.

Speculations concerning an inhibitory counterpart (N_i/G_i) for N_s evolved from the observation that hormonal inhibition of adenylate cyclase activity in the presence of α_2-adrenergic agonists, expressed a requirement for GTP which was analogous to that for hormonal stimulation (Jacobs et al., 1978). Moreover, like N_s, the putative N_i unit appeared to act as a substrate for an AB toxin. Katada and Ui (1979) showed that islet-activating protein, one of the toxins elaborated by Bordetella pertussis, suppresses the hormonal inhibition of adenylate cyclase, while often enhancing the effect of stimulatory input.

N_s and N_i have been purified to homogeneity from numerous sources (Hanski and Gilman, 1982; Hanski et al., 1981; Northup et al., 1980; Sternweis et al., 1981; Bokoch et al., 1983). Polyacrylamide gel electrophoresis shows that N_s consists of two polypeptides, occurring in one to one stoichiometry. The larger polypeptide exists in a 45K and a 52K form which differ by the alteration of two and the deletion of fourteen amino acid residues (Gilman et al., 1986). The smaller component is a 35K polypeptide. The 45/52K proteins contain the GTP-binding site and are the substrates for ADP-ribosylation by cholera toxin. Purification of N_i shows that it is comprised of 41K and 35K subunits of which the 41K unit is the substrate for ADP-ribosylation by pertussis toxin. Peptide maps generated by the proteolytic degradation of the 35K subunits from N_s and N_i, as well as studies of amino acid composition, suggest that these components are identical between the two G-proteins (Manning and Gilman, 1983). The 41K unit of N_i and the 52/45K units of N_s have been designated the α subunits, while the 35K units are known as the β subunits. A third subunit, having a molecular weight of 5 to 10K, has recently been identified (Bokoch et al., 1984; Hildebrandt et al., 1984). This protein, which may also be indistinguishable among the G-proteins, has been designated the γ subunit.

Studies of the activation of chromatographically resolved N_s and N_i by fluoride and the non-hydrolyzable guanine nucleotide analogue, guanosine 5'-3-thio-triphosphate (GTPγS), show that activation is associated with the dissociation of the 45K (Northup et al., 1982; Northup et al., 1983a; Northup et al., 1983b) or the 41K (Bokoch et al., 1983) subunits from the intact G-regulatory oligomer. These conclusions were based on the finding that guanine nucleotides or fluoride treatment allow resolution of activated α subunits which can reconstitute either stimulatory or inhibitory adenylate cyclase activity to cyc^- S49 cells. Although the reversal of α activation achieved in this manner occurs only slowly, it can be enhanced by the addition of the resolved β subunit. Notably, the activated G-protein and the resolved α subunit display similar hydrodynamic characteristics (ie. N_i and N_s behave as smaller particles upon activation than they do in their resting states).

According to Rodbell's "Disaggregation Theory of Hormone Action" (Rodbell, 1980), G-proteins, in their resting states, probably exist in an oligomeric form, complexed with an appropriate receptor and with a molecule of GDP bound at their active sites. Agonist binding induces a conformational change in the receptor which affects its relationship with the G-protein, presumably converting the guanine nucleotide-binding site from the closed to the open form. GTP is thus free to bind to the active site, displacing the previously bound GDP. GTP binding is associated with dissociation of the α subunit from the βγ dimer, a process which transforms the ligand receptor from the high to the low affinity state. The free α subunit then presumably interacts with its target enzyme. Deactivation of the coupling of the G-protein to its effector enzyme occurs upon the hydrolysis of GTP to GDP by the intrinsic GTPase activity of the α subunit. The GDP-bound α subunit reassociates with the βγ subunits to regenerate the inactive complex in which the guanine nucleotide is non-exchangeable. Such a theory accounts for the desensitization phenomena common to G-protein-mediated responses, as well as the fact that isolated ground state receptor structures display a higher molecular weight than their activated counterparts. It also accounts for the guanine nucleotide-induced dissociation of chromatographically resolved G-proteins into their constituent subunits, a process which is readily reversible in the case of GTP, but prolonged in the presence of non-hydrolyzable guanine nucleotide analogues.

It is now becoming apparent that N_s and N_i are members of a family of guanine nucleotide-binding proteins or G-proteins which exist within the matrix of the plasma membrane of most cells. These proteins display a considerable amount of homology in that all consist of trimers of α, β, and γ subunits. Whereas the 35K β and perhaps also the 10K γ subunits appear to be indistinguishable among various members of the G-protein family, the α subunits display differences with respect to molecular weights, susceptibility to ADP-ribosylation by AB toxins, isoelectric points, patterns of proteolytic digestion by Staph. aureus V8 protease, and immunological characteristics (Kanaho et al., 1986). Currently there are five recognized G proteins: N_s, the intermediary unit involved in adenylate cyclase activation; N_i , the unit involved in adenylate cyclase inhibition; transducin (T), the activator of cGMP phosphodiesterase in retinal rod outer segment; N_o, a protein of as yet unknown function; and N_n (alternatively designated N_c or N_p), the G-protein involved in neutrophil activation. N_n is a protein with an α-subunit of 40 to 41 K which is ADP-ribosylated by pertussis toxin, a reaction which inhibits its participation in the signal transduction pathway. In its activated state, the free α subunit presumably interacts with its target enzyme, a calcium-dependent phosphoinositide-specific phosphodiesterase (phospholipase C), so decreasing the calcium requirement of the enzyme to physiological ranges (Smith et al., 1986). The exact molecular mechanisms remain to be elucidated.

The idea of G-protein involvement in the signal transduction process of human neutrophils evolved from a number of studies addressing the effects of GTP and its non-hydrolyzable analogues on membrane preparations, cell homogenates, and permeabilized cells. Koo et al. (1983) reported that non-hydrolyzable GTP derivatives converted a proportion of the high affinity receptors for fmet-leu-[^{3}H]phe to the low affinity state, apparently by causing an acceleration in the rate of ligand dissociation from the receptors, an observation suggestive of G-protein involvement in the stimulus-response coupling of the chemoattractant receptor. The reversibility of this desensitization phenomenon indicates interchangeability of the high and low affinity receptor subtypes. As guanine nucleotide-induced decreases in receptor affinity are a well-documented phenomena for receptors involved in the stimulatory and inhibitory adenylate cyclase pathways (Maguire et al., 1976), a role for G-proteins in the transduction of calcium-mobilizing stimuli was likewise suspected.

Gomperts (1983) reinforced this theory by a study in which GTP and its nonhydrolyzable analogues were introduced into mast cells through permeability lesions induced in the plasma membrane upon exposure of cells to micromolar concentrations of ATP^{4-}. Upon subsequent closure of the lesions by chelation of ATP^{4-} with Mg^{2+}, Gomperts was able to demonstrate that guanine nucleotide-loaded cells exhibited an exocytotic degranulation response upon addition of calcium to the medium.

An additional study performed by Hyslop et al. (1984) demonstrated a GTPase activity in neutrophil homogenates which was stimulatable by fmet-leu-phe. Huang and Oshana (1985) showed that the presence of GTP enhanced the tyrosine phosphorylation of certain 60-67K proteins in fmet-leu-phe-stimulated plasma membranes. Interestingly, GDP and GTP actually inhibited this phosphorylation activity in resting cells.

However, speculation as to the actual position and function of the G-protein in the neutrophil transduction pathway awaited the use of pertussis toxin, an AB toxin known to ADP-ribosylate and concomitantly inactivate the α subunit of certain G-proteins such as N_i and transducin. Pertussis toxin inhibition of chemoattractant-induced calcium mobilization was reported by Molski et al. (1984). Okajima and Ui (1984) and Bokoch and Gilman (1984) demonstrated that treatment of neutrophils with pertussis toxin suppressed superoxide production, arachidonic acid release, and granular enzyme release. Similar attenuation was subsequently reported for Na^+/H^+-antiport, 46K protein phosphorylation (Volpi et al., 1985), actin association with the cytoskeleton (Shefcyk et al., 1985), chemotaxis, aggregation, and shape change (Lad et al., 1985a). As responses to the calcium ionophore, A23187, and the protein kinase C-activating phorbol ester, phorbol myristate acetate, were unaffected by pertussis toxin (Okajima and Ui, 1984; Bokoch and Gilman, 1984; Verghese et al., 1985a; Volpi et al., 1985), an early event in the transduction process, prior to protein kinase C activation and calcium mobilization, was implicated.

Although pertussis toxin inhibition was not attributable to alterations in cellular levels of cyclic AMP or decreased ligand receptor binding, it was related to the ADP-ribosylation of a membrane protein of about 41K (Okajima et al., 1984). Okajima et al. (1985) later demonstrated pertussis toxin inhibition of fmet-leu-phe-stimulated GTPase activity. Pertussis toxin was also found to abolish the regulatory effects of GTP on fmet-leu-phe receptor binding, as well as on fmet-leu-phe-promoted $[^3H]$Gpp(NH)p exchange (Lad et al., 1985b). These findings suggested the presence of a G-protein interposed between chemotactic peptide receptors and the phosphoinositide-specific phosphodiesterase. The anticipated inhibitory effect of pertussis toxin on chemoattractant-induced phosphoinositide turnover was subsequently demonstrated as a prevention of PtdIns(4)P and PtdIns(4,5)P_2 hydrolysis (Volpi et al., 1985; Verghese et al., 1985a; Ohta et al., 1985) and diminished inositol phosphate production (Bradford and Rubin, 1985b; Krause et al., 1985; Ohta et al., 1985) in response to fmet-leu-phe stimulation. A recent report (Verghese et al., 1986), demonstrating that the 40-41K G-protein of neutrophils, monocytes, and HL-60 cells is ADP-ribosylated by cholera toxin, as well as by pertussis toxin, suggests that N_n is a substrate for both of the commonly used AB toxins.

Cockcroft and Gomperts (1985) reported that GTPγS stimulated the PtdIns(4,5)P_2-specific phosphodiesterase of neutrophil membrane preparations. Barrowman et al. (1986) subsequently demonstrated guanine nucleotide-induced degranulation responses from neutrophils permeablized with Sendai virus. Smith et al. (1986) showed that, in neutrophil membrane preparations, the addition of GTP enhanced fmet-leu-phe-induced PtdIns(4,5)P_2 breakdown. The non-hydrolyzable guanine nucleotide analogue, GTPγS, was found to promote PtdIns(4,5)P_2 breakdown of a similar magnitude to that produced by fmet-leu-phe in combination with GTP.

The G-protein involved in neutrophil activation was originally thought to be identical to N_i of the inhibitory adenylate cyclase pathway. Such a proposal was based on their similar molecular weights and their susceptibility to ADP-ribosylation by pertussis toxin. The observation that purified N_i from rat brain increased fmet-leu-phe binding affinity and receptor-stimulated GTPase activity upon reconstitution into membranes from pertussis toxin-treated neutrophils (Okajima et al., 1985) was also advanced as evidence in support of this theory. The attenuation of forskolin-stimulated adenylate cyclase activity by fmet-leu-phe in neutrophil membrane preparations (Lad et al., 1985b) appeared to indicate a potential for reactivity of the chemoattractant receptor-coupled G-protein with the adenylate cyclase, as well as the phosphoinositide turnover, system.

However, coupling of the fmet-leu-phe receptor with N_i in membrane preparations does not necessarily imply that this interaction is physiologically relevant. The observation that β-adrenergic receptors (Asano et al., 1985) and rhodopsin (Cerione et al., 1985) can couple with N_i in reconstituted systems suggests that receptors and G-proteins are capable of certain interactions under experimentally contrived conditions, beyond those functionally exploited by the intact cell. Moreover, biochemical and immunochemical analysis procedures have now demonstrated important differences between N_n and N_i. Gierschik et al. (1986) used an immunochemical approach to demonstrate that N_n is distinct from the other known pertussis toxin substrates: N_i, N_o, and transducin (T). Using affinity purified antibody specific to N_o-α, they were unable to detect any labelling in immunoblots of neutrophil membranes. Moreover, an antiserum generated against T which exhibited cross-reactivity with N_i-α detected less than 10% of the total pertussis toxin substrate in neutrophils, as estimated by [^{32}P]ADP-ribose incorporation in the presence of pertussis toxin or measurement of the amount of β subunit by quantitative immunoblotting, assuming that the contribution of N_s to the total β subunit population is negligible

(Codina et al., 1985). It thus appears that N_i represents only a small proportion of the total pertussis toxin substrate, the major substrate being a novel, immunochemically distinct protein.

Furthermore, although the presence of a G-protein transducing unit between "calcium-mobilizing" receptors and the phosphoinositide-specific phosphodiesterase appears to be ubiquitous, there is evidence that the biochemical characteristics of this protein may differ among tissue types. Notably, while pertussis toxin's inhibitory effects on signal transduction in neutrophils, mast cells (Nakamura and Ui, 1983, 1985), and adipocytes (Moreno et al., 1983) are well-acknowledged phenomena, studies in hepatic (Blackmore et al., 1985), cardiac, and astrocytoma cells (Masters et al., 1985) have failed to demonstrate any pertussis toxin-mediated inhibition of phosphoinositide turnover or its resultant physiological responses.

PHOSPHOINOSITIDE TURNOVER

The discovery of hormonally-induced phosphoinositide turnover is ascribed to Hokin and Hokin (1953, 1955) who noted the increased incorporation of $^{32}P_i$ into the phosphatidylinositol component of pancreatic cell phospholipids following exposure to acetylcholine. Although, at this point, mechanistic relationships were obscure, the Hokins suspected that their observation was correlated with the ability of acetylcholine to elicit a secretory response from these cells. It is now known that the increased $^{32}P_i$ incorporation observed by the Hokins was actually the result of phosphoinositide resynthesis following an initial receptor-mediated hydrolysis of these lipids. Reports of enhanced $^{32}P_i$ and myo-[2-^{3}H]-inositol incorporation into the phosphoinositide component of phagocytosing neutrophils date back to the 1960's and 70's (Karnovsky and Wallach, 1961; Sastry and Hokin, 1965; Tou and Stjernholm, 1974). The ability to evoke phosphoinositide turnover has now been recognized for a number of chemical stimuli, including the biogenic amines, noradrenaline (Prpic et al., 1982; Uchida et al., 1982), 5-hydroxytryptamine (Fain and Berridge, 1979), and histamine (Jones et al., 1979); the peptides, vasopressin (Creba et al., 1983), substance P (Hanley et al., 1980), caerulein (Putney et al., 1983), thyrotropin-releasing hormone (Martin, 1983), platelet-derived growth factor (Habenicht et al., 1981), angiotensin (Billah and Michell, 1979), and pancreozymin (Calderon et al., 1980), the sugar, glucose (Laychock, 1983), the nucleotide, ADP (Leung et al., 1983), the eicosanoid, leukotriene B_4 (Bradford and Rubin, 1985), and the lipid derivative, platelet-activating factor (Billah and Lapetina, 1983), as well as the physical stimulus, light (Brown et al., 1984).

Phosphatidylinositol (PtdIns) is a comparatively minor phospholipid (less than 10% of total phospholipids) which is found principally in the inner leaflet of the mammalian cell membrane. The polyphosphoinositides, phosphatidylinositol 4-phosphate [PtdIns(4)P] and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] each constitute 1 to 2% of the total inositol phospholipids. They are formed by the sequential phosphorylation of phosphatidylinositol at the 4-hydroxyl, followed by the 5-hydroxyl, position of the inositol head group (reviews Hawthorne and Pickard, 1979; Irvine, 1982; Downes and Michell, 1982). The polyphosphoinositides can be converted back to phosphatidylinositol by the action of phosphomonoesterases. The concerted action of the phosphoinositide-specific kinases and phosphomonoesterases are responsible for the strict control of the size of the polyphosphoinositide pool in resting cells. It is worth observing that two independent polyphosphoinositide pools appear to co-exist in the plasma membrane: a metabolically stable hormone-insensitive pool and a labile hormone-sensitive one. Depending on the cell type, the hormone-sensitive pool may represent from 8 to 17% of the total phosphoinositide component (Fain and Berridge, 1979; Monaco, 1982).

In 1974, Hokin-Neaverson reported that the primary event in the phosphoinositide turnover response was a net loss of phosphatidylinositol with the production of an approximately equimolar amount of phosphatidic acid. These findings were initially ascribed to the phosphodiesteratic cleavage of phosphatidylinositol to diacylglycerol, a product which was subsequently phosphorylated to phosphatidic acid. Agonist-induced degradation of phosphoinositides into inositol phosphate metabolites was also gaining recognition (Durell et al. 1968). The role of polyphosphoinositides in the phosphodiesteratic reaction was demonstrated by Abel-Latif et al. (1977) who showed that the hydrolysis of PtdIns(4,5)P_2 occurred in iris smooth muscle upon challenge with acetylcholine. The coincidence between the ability of hormonal stimuli to evoke phosphoinositide turnover and their efficacy as calcium mobilizers led Michell (1975) to propose that the catabolism of these phospholipids might be an initiating factor in the mechanism of calcium elevation.

The first report of chemotactic factor-stimulated phosphoinositide degradation in the neutrophil originated in the work of Cockcroft (1980, 1981, 1982) who observed that exposure of cells to fmet-leu-phe resulted in a loss of ^{32}P radioactivity from PtdIns which was accompanied by a corresponding rise in phosphatidic acid levels. The fact that this response was calcium-requiring and could be elicited by the calcium ionophore, A23187, led these investigators to suggest that phosphoinositide turnover was a consequence of calcium mobilization. However, these conclusions, based on total phosphoinositide degradation and the generation of phosphatidic acid, a secondary product of phosphoinositide breakdown, were inadequate to assess immediate temporal changes among the various inositol phospholipids. Furthermore, a critical error was made in confounding calcium dependency with calcium regulation.

Michell et al. (1981) and Kirk et al. (1981) showed that, in hepatocytes activated by vasopressin, a Ca^{2+}-independent decrease in PtdIns(4,5)P_2 levels precedes the subsequent loss of PtdIns. In a study of the action of 5-hydroxytryptamine on insect salivary glands, Berridge (1983) demonstrated that the primary substrate of hormonally-stimulated phosphoinositide turnover was not PtdIns, but PtdIns(4,5)P_2 and perhaps also PtdIns(4)P. Whereas the degradation products of the polyphosphoinositides, $InsP_2$ and $InsP_3$, were found to increase several fold within the first 5 sec of stimulation, no significant change in PtdIns was noted until 1 min after stimulation. The rapid and transient nature of $InsP_2$ and $InsP_3$ production led him to suggest that either of these metabolites might serve as a second messenger in the mobilization of calcium from internal stores. Furthermore, Berridge et al. (1984) observed that 5-hydroxytryptamine-induced $InsP_3$ production displayed no detectable lag, whereas a delay of at least 1 sec typically preceded the calcium-dependent physiological response. The concept of phosphoinositide turnover as a transduction mechanism in calcium mobilization thus began to emerge.

It is now well acknowledged that, upon receptor occupation by "calcium-mobilizing" stimuli, activation of a phosphoinositide-specific phosphodiesterase results in the cleavage of PtdIns(4,5)P_2 into 1,2-diacylglycerol and inositol 1,4,5-trisphosphate ($InsP_3$). Two alternative approaches have been applied to the study of phosphoinositide turnover in the neutrophil. Using ^{32}P labelled cells, Cockcroft et al. (1980, 1982a,b, 1985) and Volpi et al. (1983) have monitored the loss of phosphoinositides and the corresponding increase in phosphatidic acid. Bradford and Rubin (1985a,b), using cells prelabelled with [^3H]inositol, monitored inositol phosphate production in response to fmet-leu-phe. $InsP_3$ production was found to peak within 15 to 30 sec, followed by slower and more sustained increases in $InsP_2$ and $InsP_1$ levels. Leukotriene B_4 was also found to elicit $InsP_3$ production, although the response to this stimulus was of smaller magnitude and required higher concentrations.

Both of the two immediate products of PtdIns(4,5)P_2 breakdown have demonstrated second messenger function, giving rise to a bifurcating signal transduction pathway. Diacylglycerol has been implicated in the activation of protein kinase C. Streb et al. (1983) demonstrated that introduction of the water soluble product, Ins(1,4,5)P_3, into permeabilized pancreatic acinar cells was associated with a calcium release response. This finding was confirmed in neutrophils by Prentki et al. (1984a) who observed that, in digitonin-permeabilized cells, Ins(1,4,5)P_3 releases calcium from an ATP- and vanadate-sensitive intracellular pool. As this site was insensitive to the mitochondrial poisons, antimycin and ruthenium red, it appeared to represent a vesicular, rather than a mitochondrial, store.

Ins(1,4,5)P_3 appeared to act by eliciting calcium efflux from this non-mitochondrial pool rather than by inhibiting its Ca^{2+}-ATPase-mediated calcium uptake process, since the mobilization response was still observed after inhibiting the Ca^{2+}-ATPase with vanadate or depleting ATP by means of a combination of glucose and hexokinase. Moreover, the time course of the Ins(1,4,5)P_3-induced calcium elevation response occurred 2 to 3 times more rapidly than that achieved by uptake blockade with vanadate or glucose hexokinase. Subsequent cell fractionation experiments showed that Ins(1,4,5)P_3 elicits calcium release from rat insulinoma microsomes, particularly those containing markers for the endoplasmic reticulum (Prentki et al., 1984b). Isolated mitochondria were not a target for the Ins(1,4,5)P_3-induced release.

Using high-specific activity ^{32}P-labelled Ins(1,4,5)P_3 as a ligand, Spat et al. (1986) demonstrated receptor sites on the endoplasmic reticulum of saponin-permeabilized neutrophils and hepatocytes. Binding was a saturable and reversible process specific for those inositol phosphates having efficacy in the release of calcium. Vicinal phosphates at the 4- and 5-position appear to be necessary for the action of Ins(1,4,5)P_3 as a calcium releaser (Irvine et al., 1984b), while the remaining phosphate at the 1-position may be associated with an increase in receptor affinity.

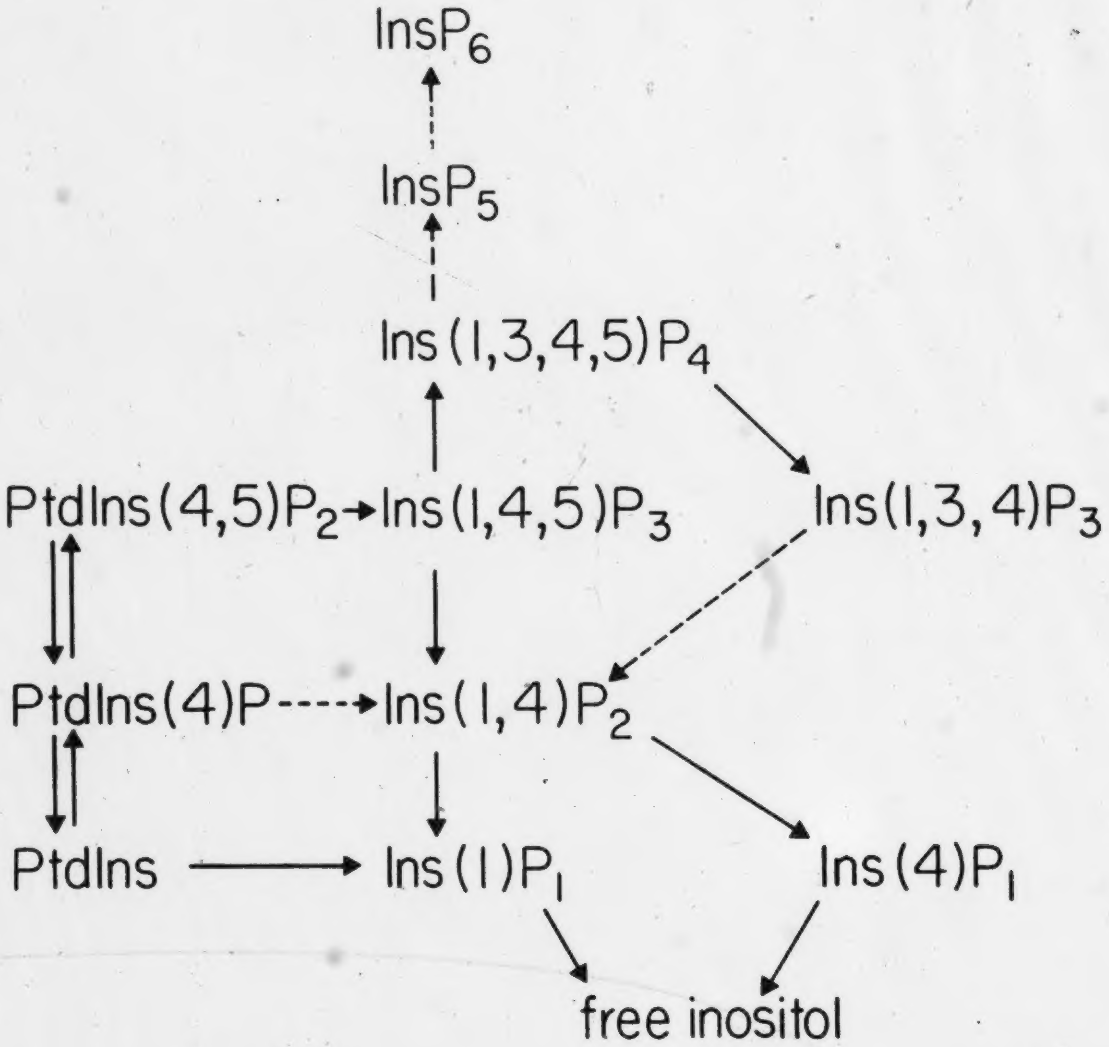
Two alternative pathways appear to be responsible for the termination of Ins(1,4,5)P_3 activity as a second messenger. A family of inositol trisphosphatases, found in the cytosol and plasma membrane, remove the phosphate from the 5-position of Ins(1,4,5)P_3 to produce Ins(1,4)P_2 which is in turn substrate for an inositol bisphosphatase which hydrolyzes it to Ins(1)P_1 (Michell, 1986). Both the 4-position-specific bisphosphatase and the inositol 1-phosphatase which reverts Ins(1)P_1 to free inositol are susceptible to inhibition by lithium (Hallcher and Sherman, 1980; Michell, 1986). However, Ins(1,4)P_2 can also be degraded to free inositol through Ins(4)P by means of a lithium-insensitive phosphatase activity (Michell, 1986). Therefore, lithium inhibition takes the form of a slowing of the inositol phosphate degradation process rather than an absolute blockade.

Alternatively, Ins(1,4,5)P_3 may be phosphorylated by an ATP-dependent kinase to inositol 1,3,4,5-tetrakisphosphate which is subsequently degraded to inositol 1,3,4-trisphosphate [Ins(1,3,4)P_3] by removal of the 5-position phosphate, probably by the same phosphatase which degrades Ins(1,4,5)P_3 (Michell, 1986). The concept of a (1,3,4) isomer of InsP_3 derived from the work of Irvine et al. (1984), performed on carbachol-stimulated rat parotid gland fragments. They discovered that the predominant InsP_3 after 15 min of carbachol stimulation was not the 1,4,5 isomer, as determined its inability to serve as a substrate of the 5'-phosphatase of human erythrocyte membrane preparations. Ins(1,3,4,5)P_4 was first detected by Batty et al. (1985), as a product generated within the first 5 sec following carbachol stimulation of muscarinic receptors in rat cortical slices. Identification was based on its chromatographic behavior, as well as its degradation to Ins(1,3,4)P_3 by the 5'-phosphatase of human erythrocyte membranes.

Neither Ins(1,3,4,5)P_4 nor Ins(1,3,4)P_3 is effectual in promoting calcium release from the endoplasmic reticulum. However, whether this pathway is merely an inactivation route for Ins(1,4,5)P_3 or whether Ins(1,3,4,5)P_4 is in fact a messenger substance in its own right, perhaps opening calcium channels in the plasma membrane (Hansen et al., 1986), remains a subject of dispute. Notably, formation of the 1,3,4-isomer of $InsP_3$ is delayed with respect to the 1,4,5-isomer, as is its degradation upon removal of stimulation (Irvine et al., 1985; Burgess et al., 1985). However, the absolute rates of synthesis and degradation of these isomers appear to be of the same order. Recent reports of an inositol pentaphosphate and hexaphosphate in GH_4 cells (Heslop et al., 1986), as well as 1,2 cyclic derivatives of $InsP_1$, $InsP_2$, and $InsP_3$ in brain (Lapetina and Michell, 1973) and sheep seminal vesicles (Wilson et al., 1985a), further complicate this scheme. Notably, Ins(1,2-cyc4,5)$InsP_3$ has been reported to mobilize calcium in permeabilized cells (Wilson et al., 1985b). A diagrammatic representation of phosphoinositide metabolism is shown in Figure 2. As the relationship of the cyclic inositol phosphates to this schema remains uncertain, these compounds have been omitted from the diagram.

Loss of the PtdIns component of membrane phosphoinositides during hormonally-stimulated metabolism was, for some time, thought to reflect, not a phosphodiesteratic breakdown event, but merely the sequential

Figure 2: Diagrammatic representation of known (continuous arrow) and probable (broken arrow) steps in phosphoinositide metabolism.


$InsP_6$
$InsP_5$
$Ins(1,3,4,5)P_4$
$PtdIns(4,5)P_2$
$Ins(1,4,5)P_3$
$Ins(1,3,4)P_3$
$PtdIns(4)P$
$Ins(1,4)P_2$
$PtdIns$
$Ins(1)P_1$
$Ins(4)P_1$
free inositol

phosphorylation of PtdIns to replenish the polyphosphoinositide stores (Berridge, 1983; Downes and Wusteman, 1983). However, although PtdIns(4,5)P_2 seems to be the initial target for phospholipase C-mediated degradation, it now appears that PtdIns may also serve as a substrate, being broken down to diacylglycerol and Ins(1)P_1. In thrombin-stimulated platelets (Wilson et al., 1985), a transient fall in PtdIns(4,5)P_2 was accompanied by an approximately 50% loss of PtdIns, while PtdIns(4)P levels were unaffected. To ascertain whether this PtdIns loss could be accounted for by polyphosphoinositide resynthesis, the rate of conversion of PtdIns to PtdIns(4)P and PtdIns(4,5)P_2 was measured by the rate of increase in specific activity occurring upon incorporation of ^{32}P into the polyphosphoinositides. Thrombin stimulation failed to change the rate of rise in the specific activity of the 4-phosphate of PtdIns(4)P. The rise in specific activity of the 5-phosphate was accelerated, however, an effect which has been interpreted both as increased phosphorylation of PtdIns(4)P (Majerus et al., 1985) and as an artifact resulting from the increase in specific activity of the γ-phosphate of ATP, secondary to a thrombin-induced acceleration of ATP turnover (Verhoeven et al., 1986). A phosphodiesteratic breakdown process thus appeared to be the only tenable explanation for PtdIns loss in thrombin-stimulated platelets. PtdIns(4)P and PtdIns(4,5)P_2 hydrolysis appear to be calcium-independent, while PtdIns hydrolysis is strictly calcium-dependent. Majerus et al. (1985) suggest that the initial PtdIns(4,5)P_2 hydrolysis incites the calcium signal which permits phosphodiesteratic cleavage of PtdIns. Diacylglycerol generation, with the resultant protein kinase C activation and arachidonic acid release, could thus proceed for some time after the cessation of $InsP_3$ generation.

PROTEIN KINASE C

Protein kinase C, is a calcium-activated, phospholipid-dependent enzyme which, under physiological circumstances, appears to be activated by 1,2-diacylglycerol (reviews Nishizuka, 1983; 1984; 1986). Although diacylglycerol is almost absent from the plasma membrane of resting cells, it is produced rapidly and transiently in response to the phosphodiesteratic cleavage of inositol phospholipids in stimulated cells. Diacylglycerol increases the apparent affinity of protein kinase C for calcium such that the enzyme can be activated at less than the 10^{-7} M physiological calcium concentration (Kishimoto et al., 1980). Full protein kinase C activation is thus possible in the absence of calcium mobilization (Kishimoto et al., 1980; Kaibuchi et al., 1981).

Triacyl- and monoacylglycerols appear to be completely ineffectual as protein kinase C activators (Kishimoto et al., 1980). Diacylglycerols functional in this capacity have the common feature of containing at least one unsaturated fatty acid, generally arachidonic acid, at position 2 (Mori et al., 1982). Neither 2,3- nor 1,3-diacylglycerols have efficacy (Rando and Young, 1982). Termination of diglyceride-mediated protein kinase C activation may occur by either of two routes: diacylglycerol may be phosphorylated to phosphatidic acid which is subsequently channelled back to the inositol phospholipids through the intermediate, cytidine diphosphate-diacylglycerol, or else further degraded by diacylglycerol lipase, a step which appears to be associated with arachidonic acid release (Berridge and Irvine, 1984). Proteolytic degradation of the enzyme molecule may also play a role in inactivation of the response (Nishizuka, 1986). Although diacylglycerols such as diolein, which contain two long chain fatty acid moieties, are not incorporated into the plasma membrane in appreciable quantities when added to cells exogenously, certain synthetic derivatives, including 1-oleoyl-2-acetylglycerol (OAG) and sn-dioctanoylglycerol, readily intercalate into the plasma membrane where they serve to activate protein kinase C in the absence of phosphoinositide turnover (Kaibuchi et al., 1982; Lapetina et al., 1985).

With respect to phospholipid cofactors, phosphatidylserine appears to be essential for protein kinase C activation, while phosphatidylethanolamine shows positive cooperativity and phosphatidylcholine and sphingomyelin show negative cooperativity (Kaibuchi et al., 1981). Like calmodulin, protein kinase C is inhibited by a number of hydrophobic, phospholipid-interacting drugs such as dibucaine, chlorpromazine, and trifluoperazine (Mori et al., 1980; Schatzman et al., 1981). The recent advent of the isoquinoline sulfonamides, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) and N-(2-aminoethyl)-5-isoquinolinesulfonamide (H-9), may provide a series of more specific protein kinase C inhibitors (Hidaka et al., 1984). Long chain sphingoid bases (Hannun et al., 1986), as well as a 17K regulator protein (McDonald et al., 1986), are now being implicated as possible endogenous inhibitors of protein kinase C.

Protein kinase C is a single polypeptide chain of molecular weight 77,000 daltons which contains two functionally distinct domains: a hydrophobic domain which has been implicated in membrane binding and a hydrophilic domain bearing the catalytic site (Kikkawa et al., 1982). Upon proteolytic cleavage of the two domains with calcium-dependent thiol proteases, a 51K fragment containing the catalytic site is produced which is fully active in the absence of calcium, phospholipid, or diacylglycerol (Inoue et al., 1977). Interestingly, these thiol proteases, which are activated at 1 to 10 µM Ca^{2+} concentrations, exhibit a preference for the activated, membrane-bound form of protein kinase C (Kishimoto et al., 1983b). Activated protein kinase C appears to have a large repertoire of protein substrates which it phosphorylates at serine and threonine residues with particular preference for those hydroxy amino acids that are located at the amino terminal end (Krebs and Beavo, 1985). Furthermore, the phosphorylated seryl and threonyl residues are generally situated with basic amino acid residues at both their amino and carboxyl sides (Kishimoto et al., 1985). Among the proteins subject to in vitro phosphorylation by protein kinase C are histone, protamine, microtubule-associated proteins, myelin basic proteins, and various membrane-bound proteins. Although protein kinase C can undergo autophosphorylation, the significance of this reaction is unknown (Nishizuka, 1986).

The complete amino acid sequence of protein kinase C has recently been elucidated by means of sequence analysis and recombinant DNA technology (Parker et al., 1986). A cysteine-rich domain, containing an internal duplication, is found at the amino terminal. At the carboxyl terminal, a catalytic domain has been identified which displays considerable homology to sequences found in other serine- and threonine-specific protein kinases. Interposed between the cysteine-rich and the catalytic regions is a domain containing a possible calcium-binding site, as suggested by its similarity to the "E-F hand" structure found in calmodulin and other calcium-binding proteins (Van Eldik et al., 1982). Studies using in situ hybridization and Southern analysis have localized a family of three genes, coding for α, β, and γ forms of protein kinase C, to distinct chromosomes in the bovine, human, and rat genome (Coussen et al., 1986). The functional disparaties between the various members of the protein kinase C family are a subject of increasing interest.

The correlation of protein kinase C activation with a physiological response was first reported in platelets. Stimulation of these cells with thrombin results in 5-hydroxytryptamine release in conjunction with the phosphorylation of a 40K protein (Lyons et al., 1975). Upon isolation, this protein can serve as a phosphorylation substrate for protein kinase C in in vitro assays. Notably, fingerprint analyses of the 40K protein following tryptic digestion show identical patterns whether it is phosphorylated by in vitro protein kinase C preparations or in vivo by thrombin-stimulated cell suspensions, suggesting the participation of protein kinase C in thrombin-mediated platelet activation (Kaibuchi et al., 1982). In a recent report, Touqui et al. (1986) show that the 40K protein, upon extraction from platelets, suppresses the activity of porcine pancreatic phospholipase A_2. This inhibitory activity was reduced in 40K protein fractions from thrombin-stimulated cells. These results, together with its cross-reactivity with monoclonal antibodies to lipocortin, have given rise to the suggestion that the 40K protein kinase C substrate in platelets may be an endogenous phospholipase A_2 inhibitor. Inactivation of the 40K substrate upon phosphorylation by protein kinase C might thus provide a possible mechanism for the arachidonic acid release response common to cells activated by "Ca^{2+}-mobilizing" stimuli (Nishizuka, 1983, 1984, 1986).

Phorbol ester tumor promotors such as phorbol 12-myristate 13-acetate (PMA) are derived from the oil of the seed of Crotum tiglium. These hydrophobic substances resemble diacylglycerol by virtue of the acyl moieties on positions 12 and 13 of their third ring. The role of PMA as a protein kinase C activator was originally suggested by studies demonstrating that, in platelets, this tumor promotor induces 40K protein phosphorylation and 5-hydroxytryptamine release, but not diacylglycerol formation (Castagna et al., 1982; Yamanishi et al., 1983). Such a role was later confirmed by in vitro studies showing that phorbol ester-mediated protein kinase C activation resembles that of diacylglycerol in exhibiting calcium- and phospholipid-dependency and involving an increase in the affinity of the enzyme for calcium and phospholipid (Kikkawa et al., 1983). However, as phorbol esters are not rapidly degraded by the endogenous enzymes which inactivate diacylglycerol, cellular responses to these agents are prolonged, perhaps accounting for their involvement in oncogenic transformations. Notably, non-tumor-promoting phorbol esters, such as phorbol 12, 13-didecanoate, are ineffectual as protein kinase C activators.

The concept of protein kinase C as the phorbol ester receptor emerged from the observations that the tritiated phorbol ester, [^{3}H]phorbol 12,13-dibutyrate binds to purified protein kinase C in a calcium- and phospholipid-dependent manner (Kikkawa et al., 1983) and competes with diacylglycerol for binding sites (Sharkey et al., 1984; Sharkey and Blumberg, 1985). Furthermore, protein kinase C activity and phorbol ester binding sites have been found to display similar patterns of tissue distribution (Ashendel et al., 1983). In an attempt to localize phorbol ester binding and protein kinase C activities to partially purified protein components of cell extracts, Niedel et al. (1983) collected the particulate fraction from rat brain cells incubated with phorbol esters and calcium, then proceeded to remove the membrane-bound receptor by chelating calcium from the medium with EDTA/EGTA buffer. Solubilized receptor was subjected to partial purification by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography. Assay of the resulting fractions for phorbol ester binding and protein kinase C activity revealed that the two functions co-eluted in a single, symmetrical peak. A more recent study, in which neutrophil protein kinase C was purified to near homogeneity, revealed the co-purification of phorbol ester binding and kinase activity throughout the chromatographic procedure (Christiansen et al., 1986). Interestingly, a number of non-diacylglycerol-like tumor promotors, including mezerein, telocidin, and aplysia toxin also serve as protein kinase C activators, although with less potency (Miyake et al., 1984; Fujinki et al., 1984). These agents have been suggested to result in changes in membrane properties analogous to those of diacylglycerol.

Although phorbol esters activate superoxide radical production (Repine et al., 1974) and specific granule release (Estensen et al., 1974) from neutrophils, it is worth recognizing that these agents actually exert negative modulatory effects on some other aspects of the phosphoinositide-dependent signal transduction pathway. For example, Lagast et al. (1984) and Schell-Frederick (1984) observed that PMA pretreatment prevented the subsequent calcium mobilization response to fmet-leu-phe. Naccache et al. (1985a) found that fmet-leu-phe- and LTB_4-stimulated degranulation were likewise inhibited by phorbol ester treatment. Sha'afi et al. (1986) noted that these inhibitory phorbol ester effects, as well as 50K protein phosphorylation, could be suppressed by the protein kinase C inhibitor, H-7, presumably indicating that the inhibition of chemoattractant effects was attributable to protein kinase C activation and not non-specific effects of the phorbol esters. Although PMA was not found to inhibit fmet-leu-phe-binding (Naccache et al., 1985a), it was found to inhibit fmet-leu-phe-stimulated GTPase activity (Matsumoto et al., 1986), a finding which implicates the G-protein as a possible site for protein kinase C-mediated inhibition of the signal transduction process.

Kraft and Anderson (1983), in a study of parietal yolk sac (PYS) cells, demonstrated that protein kinase C activation appears to be associated with intracellular redistribution of the enzyme. Upon disruption of PYS cells by homogenization, the cytosolic fraction was collected in the form of the 100,000 X g supernatant. The 100,000 X g pellet, representative of the membrane fraction, was subjected to detergent solubilization and re-centrifugation. Assay of the resulting fractions for calcium- and phospholipid-dependent kinase activity revealed that protein kinase C in untreated PYS cells was localized predominantly in the cytosolic fraction, while that in the membrane fraction was negligibly low. However, in cells exposed to phorbol ester tumor promotors prior to homogenization and fractionation, protein kinase C activity was discovered to be drastically diminished in the cytosolic fraction and correspondingly increased in the membrane fraction. This phenomenon was recently confirmed in neutrophils by Wolfson et al. (1985) using the phorbol ester, PMA, to achieve translocation of the kinase. Significantly, over two-thirds of PMA-induced protein kinase C redistribution preceded NADPH oxidase activation, a finding which is consistent with the idea that protein kinase C translocation is a necessary precedent to superoxide production. It thus seems that the tight, rapid association of kinase C with the plasma membrane is an early event in its activation process.

Exactly how this redistribution process occurs is yet uncertain. However, one suggestion is that phorbol esters somehow mediate the translocation of soluble, inactive protein kinase C from the cytosol to the membrane where it is activated. An alternative and perhaps more appealing hypothesis suggests that protein kinase C exists in a state of loose association with the plasma membrane. Hydrophobic phorbol esters readily penetrate the plasma membrane and somehow intensify the kinase's association with the membrane (Kraft and Anderson, 1983). This may occur due to phorbol ester-mediated changes in membrane structure or composition. Another possibility is that the intercalated phorbol ester favours the formation of a quaternary complex with protein kinase C, calcium, and phospholipid (Nishizuka, 1984). Helfman et al. (1983) reported that the apparent cytosolic localization of protein kinase C in unstimulated cells is probably an artifact resulting from calcium chelation by the EDTA-containing buffer used during cell disruption. When cells were homogenized in the absence of chelators, the enzyme expressed a weak affinity for the membrane fraction, suggestive of a plasma membrane protein.

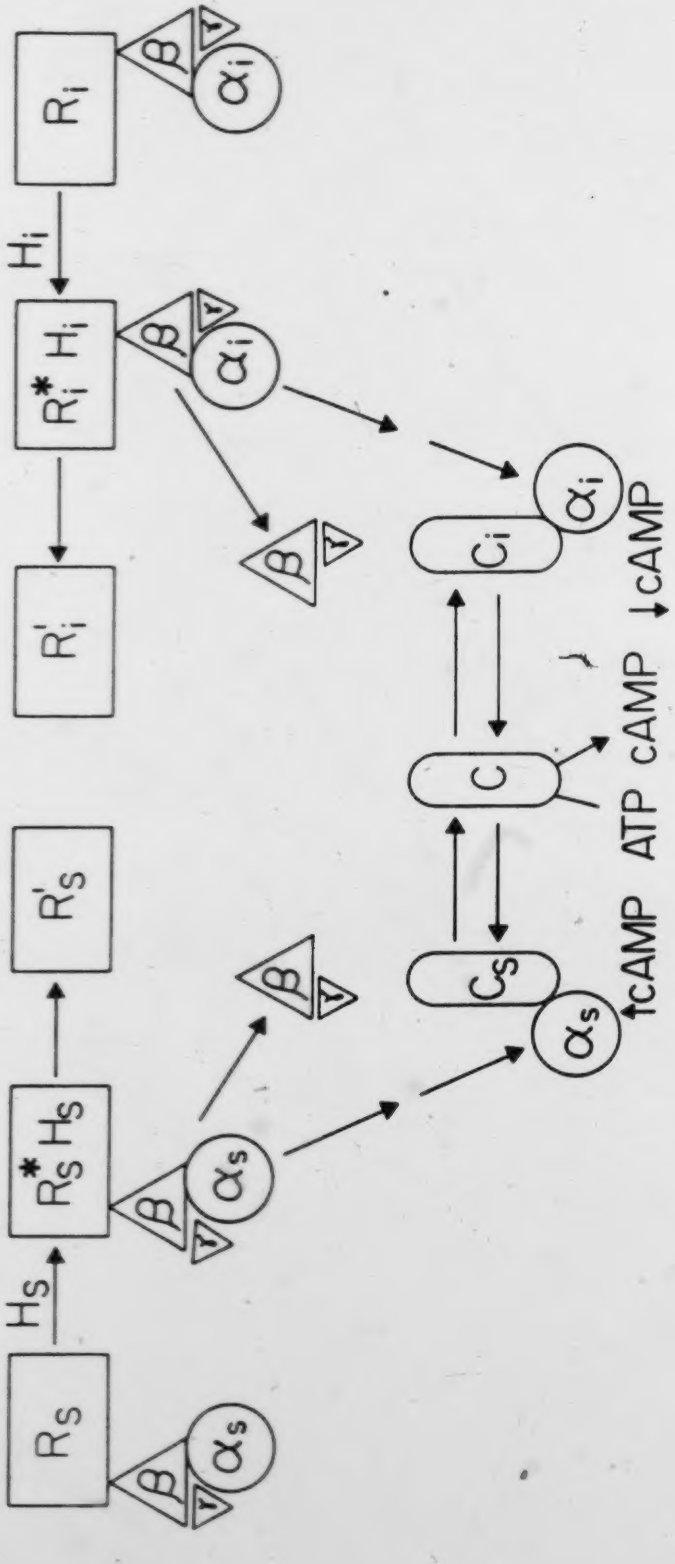
ADENYLATE CYCLASE IN THE NEUTROPHILS

The neutrophil plasma membrane is equipped with adenylate cyclase, the enzyme responsible for the cyclization of adenosine triphosphate (ATP) to the second messenger substance, cyclic AMP. Cyclic AMP (cAMP) is responsible for the activation of protein kinase A, an enzyme which, in its resting state, exists as a tetrameric complex of two regulatory and two catalytic subunits. Binding of cAMP to the regulatory dimer of the enzyme promotes dissociation of the catalytic dimer into the free, active monomeric subunits which are responsible for phosphorylation of the target proteins (review Hoppe, 1985).

Adenylate cyclase is subject to dual stimulatory and inhibitory hormonal input, through coupling with the mutually antagonistic G-protein transducing units, N_s and N_i. Cyclase activation during interaction with N_s is associated with a decrease of the enzyme's magnesium requirements to physiological levels (Jakobs et al., 1983). Whether N_i-induced inhibition is due to direct interaction of the α-subunit with the catalytic moiety or a β-subunit-mediated shift in equilibrium favouring the undissociated form of N_s remains a matter of dispute. In support for the former theory is the finding that N_i-mediated inhibition affects not only guanine nucleotide- and hormone-stimulated adenylate cyclase (Jakobs et al., 1985a), but also cyclase activated by forskolin, a direct stimulator of the catalytic unit (Daly, 1984). Furthermore, hormonal inhibition of basal adenylate cyclase activity has been observed in cyc^- membranes which lack a functional N_s (Hildebrandt et al., 1982; 1983). Nevertheless, Smigel et al. (1984) have shown that the chromatographically resolved 35K subunit of N_i accelerated the deactivation of fluoride-activated N_s, whereas the 41K N_i subunit actually stabilized N_s in the activated form. The 41K subunit was, however, found to have weak inhibitory effects on the catalytic moiety, in contrast to the 35K subunit, whose inhibitory effects were strictly dependent on the presence of activated N_s. It thus seems likely that both the α and β subunits of N_i may contribute to hormonally-induced inhibition of adenylate cyclase. A schematic representation of some of the major events in cyclase regulation is shown in Figure 3.

In the neutrophil, inhibition of acute chemoattractant-induced inflammatory responses is associated with the elevation of intracellular cyclic AMP levels either through incubation of cells with the lipid-permeable cyclic nucleotide analogue, dibutyryl cyclic AMP, or upon activation of the adenylate cyclase system by means of forskolin, β-adrenergic agonists or prostaglandins of the E or I series. Inhibition has been observed at the level of phosphoinositide turnover (Takenawa et al., 1986; Della Bianca et al., 1986a), calcium mobilization (DeTogni et al., 1984), degranulation (Simchowitz et al., 1980), and superoxide

Figure 3: Schematic representation of the molecular events involved in adenylate cyclase activation (H_s = stimulatory hormone, H_i = inhibitory hormone, R_s = stimulatory receptor, R_i = inhibitory receptor, $R_s^*H_s$ = activated complex of stimulatory hormone and receptor, $R_i^*H_i$ = activated complex of inhibitory hormone and receptor, R_s' = desensitized stimulatory receptor, R_i' = desensitized inhibitory receptor, α_s = α subunit of N_s, α_i = α subunit of N_i, β = β subunit, γ = γ subunit, C = catalytic subunit of cyclase, C_s = stimulated catalytic subunit of cyclase, C_i = inhibited catalytic subunit of cyclase, ATP = adenosine triphosphate, cAMP = cyclic adenosine monophosphate).


R_S
β
γ
α_s
H_S
R*_S H_S
R'_S
R_i
H_i
R*_i H_i
R'_i
α_i
C_S
C
C_i
↑cAMP
ATP
cAMP
↓cAMP

production (Wong and Freund, 1981). Various reports have attributed this inhibition to either a reduction in the number of chemotactic receptor binding sites (Holian et al., 1986) or inhibition of phospholipase C activity (Snyderman et al., 1986). Notably, the inhibitory effects of cyclic AMP are manifested only with respect to receptor-mediated inflammatory stimuli and appear not to influence inflammatory responses induced by fluoride ion or the phorbol ester tumor promotor, PMA (Wong, 1983, Fujita et al., 1984).

It is then, perhaps, rather paradoxical that a number of chemotactic stimuli such as the formylated tripeptide, fmet-leu-phe, and the complement factor, C5a, induce a transient elevation of intracellular cyclic AMP levels during the course of neutrophil activation (Jackowski and Sha'afi, 1979; Simchowitz et al., 1980). However, the early suggestion that chemoattractant receptors might have direct interactions with N_s was contradicted by the finding that, unlike β-adrenergic agonists or prostaglandins of the E series, fmet-leu-phe failed to stimulate adenylate cyclase activity in neutrophil membrane preparations (Verghese et al., 1985b). Neither did it inhibit prostaglandin E_1-induced cyclase stimulation (Verghese et al., 1985b), although forskolin-induced stimulation was somewhat inhibited (Lad et al., 1985b; Saad et al., 1986). Moreover, while prostaglandin- or adrenergic agonist-induced cAMP production in intact cells was independent of calcium, that activated by chemoattractants proved to be calcium-requiring, being reduced under circumstances of extracellular calcium depletion (Jackowski and Sha'afi, 1979; Verghese et al., 1985) and almost totally inhibited by 8-(N,N-diethylamino)-ocytyl-3,4,5-

trimethoxy-benzoate (TMB-8), a drug which blocks calcium influx in neutrophils (Verghese et al., 1985). Furthermore, while prostaglandin E_1-induced cyclase stimulation was inhibited by N_i activation through α-adrenergic agonists, the fmet-leu-phe-induced cAMP response was unaffected, suggestive of a mechanism of cyclase activation independent of G-protein involvement.

The observation that 5-lipoxygenase inhibitors suppress chemoattractant-induced cAMP elevation suggests that perhaps this response is attributable to the generation of some eicosanoid metabolite, such as LTB_4 (Hopkins et al., 1983). An alternative suggestion (Verghese et al., 1985b) is that chemoattractants may inhibit phosphodiesterase activation. However, these hypotheses are, as of yet, controversial. Although it has been postulated that chemoattractant-induced elevation of cyclic AMP levels may serve as a form of negative feedback regulation (Verghese et al., 1985b), other reports tend to indicate that the magnitude of the chemoattractant-induced cyclic AMP response does not attain the threshold at which inhibitory effects become expressed (Smolen et al., 1980).

II. OBJECTIVES

In an effort to learn more about the transduction of inflammatory stimuli in human neutrophils, pharmacological probes were used to achieve activation and suppression of cellular responses at various levels of the transduction pathway. A series of studies, involving neutrophil activation through the use of a phorbol ester, a calcium ionophore, guanine nucleotide analogues, and fluoride ion, were performed in attempts to elucidate critical events in the regulation of inflammatory responses at the biochemical level.

(1) Synergistic interactions between the calcium mobilization and protein kinase C branches of the bifurcating phosphatidylinositol pathway of cell activation were first reported by Kaibuchi et al. (1982, 1983) and Yamanishi et al. (1983) who demonstrated that the calcium ionophore, A23187, at concentrations which were not in themselves stimulatory, synergistically enhanced 5-hydroxytryptamine release from platelets undergoing degranulation in response to submaximal concentrations of the synthetic diacylglycerol, 1-oleoyl-2-acetyl-glycerol, and the phorbol ester tumor promotor, phorbol 12-myristate 13-acetate (PMA). These findings were later extended to lysosomal enzyme release from neutrophils (Kajikawa et al., 1983) and histamine release from mast cells (Katakami et al., 1984).

In an attempt to extend these observations to the superoxide production response of human neutrophils, oxygen radical release from neutrophils stimulated with PMA and A23187, alone and in combination, was investigated and quantified with respect to lag period, superoxide generation rate, and total superoxide production using a spectrophotometric assay technique. Toxic effects at high concentrations of the ionophore were also addressed.

(2) Phorbol ester-induced desensitization of hormone-stimulated adenylate cyclase activation has been reported for the action of glucagon on hepatocytes (Heyworth et al., 1984) and for β-adrenergic agonists on avian erythrocytes (Sibley et al., 1984; Kelleher et al., 1984). In avian erythrocytes, this desensitization has been correlated with the phosphorylation of β-adrenergic receptors, presumably due, either directly or indirectly, to phorbol ester-mediated activation of protein kinase C.

On the basis of these reports, it was desirable to determine whether phorbol ester inhibition of agonist-stimulated adenylate cyclase activity applied to fmet-leu-phe and PGE_1 effects on the neutrophil. This problem was approached through a quantification of the agonist-induced cAMP production in phorbol ester-treated and untreated cells by means of a radioimmunoassay procedure.

(3) Guanine nucleotide regulation of fmet-leu-phe binding (Koo et al., 1983), as well as pertussis toxin inhibition of various chemoattractant-mediated cellular effects (Okajima and Ui, 1984; Bokoch and Gilman, 1984), have led to the concept of G-protein involvement in the neutrophil signal transduction pathway. In an effort to demonstrate a positive relationship between guanine nucleotides and a cellular response, the effect of non-hydrolyzable guanine nucleotide analogues on granular enzyme release was investigated. Non-hydrolyzable GTP analogues, by virtue of their resistance to the GTPase activity of the α unit which normally terminates G-protein activation, lead to a

persistently activated state of these transducing units (Schramm and Rodbell, 1975). However, as nucleotides are impermeant at the level of the cell membrane, it was necessary to implement a permeabilization procedure, utilizing the detergent saponin, in order to gain access to the intracellular space. Degranulation from the nucleotide-loaded cells was examined with respect to calcium- and nucleotide-dependency using a spectrophotometric assay procedure.

(4) The ability of fluoride to activate a respiratory burst was first recognized by Sbarra and Karnovsky (1959) who noted an increased oxygen uptake response in cells which were being exposed to the ion as an enolase inhibitor. Selvaraj and Sbarra (1966) advanced the idea that this inflammatory effect was attributable to a hypothetical insoluble precipitate formed by the interaction of fluoride with other ions within the cells. Fluoride-induced superoxide production was subsequently documented in a number of studies (Curnutte and Babior, 1975; Curnutte et al., 1977; Harvath et al., 1978). Curnutte et al. (1979) made some attempts to characterize this response: they found that, although the fluoride-response was independent of K^+ or Mg^{2+}, there was a strong Ca^{2+} requirement. Other halide ions were found to be ineffective as inflammatory stimuli. Wong (1983) addressed the interactive effects of fluoride and fmet-leu-phe. The chemotactic peptide was found to potentiate the slower response to fluoride by decreasing the lag period, as well as accelerating the rate at suboptimal temperatures, phenomena which led him to conclude that the mechanisms of action of the two stimuli involved a common component.

Howlett et al. (1979) observed that fluoride, like the non-hydrolyzable guanine nucleotide analogues, Gpp(NH)p and GTPγS, elicited a persistent state of adenylate cyclase activation in membrane preparations from S49 lymphoma cells. Stimulation of cyclase activity by these agents could not be observed in the purified catalytic moiety. However, upon addition of a second protein, which was retained on GTP affinity supports and released by Gpp(NH)p, the responsiveness of the cyclase to fluoride (plus Al^{3+}) and the non-hydrolyzable guanine nucleotide analogues was restored. Activation was found to be related to the dissociation of N_s into its 45 K and 35 K subunits. The ability of fluoride to activate N_i and transducin was subsequently reported (Katada et al., 1984a,b; Stein et al., 1985).

Although the ability of fluoride to activate a respiratory burst (Curnutte et al., 1979), arachidonic acid release (Bokoch and Gilman, 1984), and proton extrusion (Takanaka and O'Brien, 1985) in the neutrophil has been recognized for some time, few attempts have been made to discern the molecular mechanism of action of this ion as an inflammatory stimulus. In view of fluoride's efficacy as an activator of N_s, N_i, and transducin, we have examined the possibility that fluoride-induced inflammatory responses may be occurring at the level of a G-protein. Were this the case, fluoride would be expected to mimic the action of "calcium-mobilizing" receptor stimuli in activating phospholipase C with the resultant degradation of phosphoinositides, as determined by the accumulation of inositol phosphate metabolites in the acid extracts of activated cells. Since the second messenger substances generated during phosphoinositide turnover are associated with calcium mobilization and protein kinase C redistribution, it was desirable to investigate these events. The elevation of intracellular calcium concentrations was assayed using the fluorescent calcium probe, Quin 2. Protein kinase C activation was studied on the basis of alterations in the level of calcium- and phospholipid-dependent protein kinase activity in the cytosolic and membrane fractions obtained from activated cells.

CHAPTER II

METHODS AND MATERIALS

A. MATERIALS

The following chemicals and supplies were obtained from the sources indicated: heparinized Vacutainer tubes, saponin (Fisher Scientific Co., Fair Lawn, N.Y.), Hanks' Balanced Salt Solution, HEPES buffer (Gibco Laboratories, Grand Island, NY), Ficoll-Paque (Pharmacia Inc., Dorvall, PQ), L-myo[1,2-^{3}H]myo-inositol, D-[inositol-2-^{3}H(N)]-1-phosphate, D-[inositol-2-^{3}H(N)]-1,4-bisphosphate, D-[inositol-2-^{3}H(N)]-1,4,5-trisphosphate, cyclic AMP radioimmunoassay (New England Nuclear, Boston, Mass.), horseheart ferricytochrome c (Type VI), phorbol myristate acetate, xanthine oxidase, ethidium bromide, Micrococcus lysodeikticus, lysozyme standard, cytochalasin B, formylmethionyl-leucyl-phenylalanine, prostaglandin E_1, cholera toxin, guanosine 5'-monophosphate, guanosine 5'-diphosphate, guanosine 5'-triphosphate, 5'-guanylylimidodiphosphate, adenosine 5'-diphosphate, adenosine 5'-triphosphate, N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate, phenylmethylsulfonyl fluoride, leupeptin, Type II-A histone, phosphatidylserine (Sigma Chemical Co., St. Louis, MO), [γ^{32}P]ATP, ACS II (Amersham, Oakville, Ontario), pertussis toxin (List Biological Laboratories, Campbell, CA), guanosine 5'-O-(3-thio-triphosphate) (Boehringer Mannheim, St. Louis, MO), and forskolin, A23187, and Quin 2/AM (Calbiochem, San Diego, CA).

All buffers were prepared using deionized distilled water which was filtered through a Millipore RQ Water Purifier. Stock solutions of the water-insoluble compounds, phorbol myristate acetate, cytochalasin B, formylmethionyl-leucyl-phenylalanine, forskolin, A23187, and Quin 2/AM, were prepared in dimethylsulfoxide. However, the dimethylsulfoxide content in reaction mixtures was never allowed to exceed 0.5% (vol/vol) (Wong and Chew, 1982). Other agents were dissolved either in distilled water or in the incubation buffer in question.

B. NEUTROPHIL ISOLATION PROCEDURE

Blood samples were obtained by venupuncture from healthy human volunteers and collected in heparinized Vacutainer tubes (Becton Dickinson, Mississauga, Ont.). Donors were of either sex and ranged in age from 18 to 60. Neutrophils were isolated as previously reported (Wong and Freund, 1980; Strnad and Wong, 1985). As neutrophils have a tendency to adhere to glass, polypropylene tubes were used for all stages in the preparation procedure (Falcon Plastic, Oxnard, CA).

Following an initial 10 min centrifugation of the whole blood at 400 X g, the plasma was removed to a point approximately 1 cm above the compacted cellular components. The blood was then diluted in Hanks' Balanced Salt Solution (HBSS, composition: 137 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 5.6 mM glucose, 1.6 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4) and layered in 8 to 10 mL volumes onto approximately 3 mL of a sterile Ficoll-sodium diatrizoate solution, Ficoll-Paque. Ficoll is a polymeric erythrocyte aggregating agent of low viscosity. During the course of a 30 min centrifugation at 400 X g, the erythrocytes, upon being aggregated by the Ficoll, sediment completely through the Ficoll-Paque. Likewise, the neutrophils, at the osmotic pressure of the Ficoll-Paque solution, achieve a density sufficient for their migration through the Ficoll layer. However, the lymphocytes and monocytes, owing to their lower density, are unable to penetrate this barrier and are found at the interface between the Ficoll-Paque and the HBSS medium (Boyum, 1968).

Following removal of the upper layers of the density gradient, the neutrophil-erythrocyte pellet was resuspended in a hypotonic salt solution (0.15 M NH_4Cl, 0.01 M $NaHCO_3$, and 0.1 mM Na_2EDTA, pH 7.2) to achieve lysis of the erythrocyte component. A 10 min centrifugation at 400 X g was used to sediment the isolated neutrophils. This step was repeated once. Finally, cells were washed twice by resuspension and centrifugation in HBSS. Cell yield was determined and cell density adjusted through use of a Coulter counter, Model ZBI high-speed electronic particle counter. Cell yield was generally in the range of 2 to 5 X 10^6 cells/mL of whole blood. This procedure typically yielded a cell suspension which was greater than 94% neutrophils, as determined by microscopic inspection. Cell viability, as assessed by Trypan Blue exclusion, was greater the 98%.

C. CYTOCHROME C ASSAY

The release of superoxide anions generated by activated neutrophils was monitored continuously by means of the increase in absorbance of the indicator species, ferricytochrome c, occurring upon its reduction by oxygen free radicals (Cohen and Chovaniec, 1978; Wong, 1983).

$$O_2^- + \text{cytochrome } c^{3+} \longrightarrow O_2 + \text{cytochrome } c^{2+}$$

Absorbance changes were followed at a wavelength of 550 nm and a slit width of 1 nm against a distilled water blank in either a Perkin Elmer (model 552) or a Shimadzu UV-240 spectrophotometer.

Reaction mixtures consisted of 0.1 mM ferricytochrome c and 1 X 10^6 cells diluted to 1 mL in Hanks' Balanced Salt Solution (HBSS). The reagents in question were added at the desired concentrations. Samples were assayed in 1-cm polystyrene cuvettes in thermostatted cell holders adjusted to 37°C. To control for possible effects of the tested agents on the ferricytochrome c reduction process, a cell-free superoxide-generating system, consisting of 0.0125 units of xanthine oxidase as the superoxide production source and 50 µM xanthine as the substrate, was used in the place of cells in preliminary studies.

Reaction progress tracings, obtained from a moving chart recorder, were used in data analysis. Lag periods were determined by extrapolating the linear portion of the reaction progress tracing to the time axis. Superoxide generation rates were determined by the division of the rate of the absorbance change (calculated as the slope of the linear portion of the tracing) by the molar extinction coefficient (21/cm/mM) for the absorbance difference between reduced and oxidized cytochrome c. Total superoxide production was determined by dividing the overall absorbance change by the extinction coefficient.

D. LYSOZYME ASSAY

Lysozyme, by virtue of its ability to hydrolyze the β-1,4-glucosidic linkages in the mucopolysaccharide cell wall of the bacterium, Micrococcus lysodeikticus, causes the lysis of this organism. Assays for lysosomal enzyme release were conducted by monitoring for the decrease in absorbance at 450 nm in a suspension of this target microorganism (Shugar, 1952). Reaction mixtures comprised

1.45 mL of a suspension of M. lysodeikticus (0.16 mg/mL) in phosphate buffer (67 mM NaH_2PO_4, 67 mM Na_2HPO_4, pH 6.7) and 0.50 mL of sample, consisting of the 15,000 X g supernatant obtained from cells pelleted through silicone oil after exposure to the stimulus in question. For the phorbol ester/calcium ionophore study, a 10 min preincubation of cells with 10 µM cytochalasin B was performed, such that granular discharge into the extracellular medium was promoted (Showell et al., 1983). This step was excluded from the studies involving permeabilized cells. Positive controls were obtained by lysing cell samples with 1% Triton X-100. A decrease in absorbance of 0.001/min at 450 nm and 25°C is representative of one unit of lysozyme activity. Samples were assayed in polystyrene cuvettes at a slit width of 1 nm against a distilled water blank in a Perkin Elmer (model 552) spectrophotometer.

E. LACTIC DEHYDROGENASE ASSAY

Lactic dehydrogenase assays were performed as previously reported by Wacker et al. (1956). As lactic dehydrogenase is a cytosolic enzyme, its release into the extracellular medium can be viewed as a criterion for cytotoxicity. Lactic dehydrogenase, in the presence of L-lactate, catalyzes the reduction of the diphosphopyridine nucleotide, β-nicotinamide adenine dinucleotide (NAD), with the concomitant generation of pyruvate:

$$\text{L-lactate} + \text{NAD} \xrightarrow{\text{lactic dehydrogenase}} \text{L-pyruvate} + \text{NADH} + H^+$$

This reaction can be monitored spectrophotometrically at a wavelength of 340 nm and a slit width of 1 nm against a blank containing 0.15 mL NAD (0.05 M, pH 7.5) and 1.35 mL distilled water. An increase in absorbance of 0.001 AU/min at 25°C is representative of one unit of lactic dehydrogenase activity.

Reaction mixtures consisted of 0.75 mL sodium phosphate buffer (0.1 M Na_2HPO_4, pH 8.8), 0.5 mL sodium lactate (0.16 M, pH 7.0), 0.15 M NAD (0.05 M, pH 7.5), and 0.1 mL of 15,000 X g supernatant from cells exposed to the treatments in question. The 15,000 X g supernatant from a suspension of cells ruptured by four 15 sec bursts at 60 Watts on a Braunosonic 1510 sonicator was used to obtain a measure of the total lactic dehydrogenase content in cells.

F. CYCLIC AMP RADIOIMMUNOASSAY

Samples for cAMP radioimmunoassays were prepared as previously described (Wong, 1983). Isolated neutrophils were diluted to 1 X 10^6 cells/mL in Hanks' balanced salt solution, then incubated at 37°C for 30 min. Upon appropriate treatment with the agents of interest, 0.5 mL aliquots of cell suspension were removed from the reaction mixtures at designated time intervals, transferred to 12 mm glass culture tubes, and subjected to heat inactivation by placing of the tubes over the flame of a bunsen burner for 2 to 5 sec time periods such that the samples started to boil. Each heat-inactivated sample was then sonicated for 15 sec at 60 Watts in a Braunosonic 1510 sonicator. Sonicated samples were then transferred to plastic conical tubes and centrifuged for 1 min in an Eppendorf microfuge at 15,000 X g. The resultant supernatants were transferred to 12 mm glass culture tubes and stored on ice until use in the cAMP radioimmunoassay. Phosphodiesterase inhibitors were not used during the sample preparation procedure.

The New England Nuclear cAMP radioimmunoassay kit used in this study employed, for the labelled antigen, a succinyl tyrosine [^{125}I]-methyl ester derivative of cAMP (ScAMP-TME[^{125}I]tracer). Non-radioactive antigen (cAMP in standards or unknown) was allowed to interact with a fixed amount of antibody in the presence of a constant amount of the labelled cAMP antigen. The antibody consisted of a pre-reacted primary and secondary antibody complex. The primary antibody was obtained from rabbits challenged with a succinyl cAMP-albumin conjugate, while the second antibody was generated in sheep, following immunization against rabbit globulin.

Owing to the small amount of cAMP present in the samples, it was desirable to acetylate both standards and unknowns to produce 2'-O-acetyl cAMP. Cyclic nucleotides substituted at the 2'-O-position are reported (Steiner et al., 1972) to express a higher affinity for the antibody. This enables them to compete more readily for binding sites with the [^{125}I]-labelled antigen than do their unsubstituted counterparts.

Standard solutions were prepared containing 0.1 to 4.0 picomoles/mL cAMP standard in 0.05 M sodium acetate buffer, pH 6.2. Aliquots of 100 µL of standard or sample were placed in paired tubes and acetylated using 5 µL of acetylation reagent, consisting of 2 volumes of triethylamine to 1 volume of acetic anhydride. Tubes were mixed immediately by vortexing for a few seconds.

A 100 µL volume of working tracer solution consisting of one volume of ScAMP-TME[^{125}I]tracer to one volume of cAMP carrier serum, was added to each tube. After the subsequent addition of 100 µL of the cAMP antiserum complex, the tubes were vortexed, covered with aluminum foil, and allowed to incubate 16 to 18 hr at 4°C.

After completion of the incubation, 1 mL of cold sodium acetate buffer was added to each tube followed by vortex mixing. Tubes were then centrifuged at 4°C for 15 min at 2,000 X g. The supernatants were decanted into a radioactive waste container after which the tubes were inverted over absorbent paper for a few minutes to facilitate draining of the last drops. The precipitate-containing tubes were then counted in a gamma counter (LKB-Wallac CliniGamma 1272) for 5 min.

To obtain a value of normalized percent bound ($\%B/B_0$), the average net counts per minute obtained for each cAMP standard or sample was expressed as a percent of the average net counts per minute obtained for the zero standard which contained antiserum complex and labelled antigen in the absence of additional unlabelled cAMP antigen.

$$\%B/B_0 = \frac{\text{Average Net Counts for Standard/Sample}}{\text{Average Net Counts for Zero Standard}} \times 100$$

A standard curve was then constructed on semi-log paper in which the $\%B/B_0$ value for each standard was plotted against the number of picomoles of cAMP added to that tube. The cyclic AMP content of each sample tube was determined by interpolation from the standard curve.

G. NEUTROPHIL PERMEABILIZATION PROCEDURE

Neutrophil permeabilization was carried out according to a modification of the procedure of Smolen and Stoehr (1985). Isolated neutrophils were suspended in Buffer K (100 mM KCl, 20 mM NaCl, 1 mM EGTA, 30 mM HEPES, pH 7), a solution designed to mimic the intracellular ionic composition, as well as to prevent premature calcium-dependent activation of the cells. Stock solutions of the cholesterol complexing agent, saponin, were prepared daily in this medium. Although saponin suspensions were agitated prior to use, no special precautions were taken to ensure homogeneity of detergent dispersion in the solution. Incubations were performed at 25°C at a cell density of 25 X 10^6/mL. Permeabilization was achieved by means of a 5 to 7 min incubation with 0.005% saponin, in the presence or absence of guanine nucleotides. At the end of the incubation, cell suspensions were diluted with excess Buffer K and sedimented by a 10 min centrifugation at 400 X g.

Cells were then resuspended in either normal HBSS or else in calcium-free HBSS containing 1 mM EGTA, both prewarmed to 37°C. Cells resuspended in calcium-containing media would be able to restore their normal intracellular Ca^{2+} levels through the action of calcium pumps on the plasma membrane. Incubations at 37°C were sustained for fixed time intervals which were terminated by a 1 min centrifugation through silicone oil at 15,000 X g in an Eppendorf microfuge. The supernatants were retained and stored on ice for use in enzyme assays.

As Mg-ATP has inhibitory effects on the saponin-permeabilized cell (Smolen and Stoehr, 1985), it was excluded from the permeabilization medium. This is in contrast to the study of Barrowman et al. (1986), in which metabolic support in the form of Mg-ATP was a requirement. However, it seems conceivable that, in permeabilized cells which have undergone partial "resealing" following resuspension in normal physiological media, endogenous processes may provide an adequate supply of ATP for support of the energy-dependent degranulation process.

H. ETHIDIUM BROMIDE TECHNIQUE FOR MONITORING CELL PERMEABILIZATION

Neutrophil permeabilization was monitored by the increase in fluorescence signal of the nuclear dye, ethidium bromide (Gomperts, 1983), upon addition of saponin. Being impermeant at the level of the plasma membrane and fluorescent only when complexed with nuclear DNA to form the ethidium-DNA fluorochrome, ethidium bromide is a suitable indicator with which to detect the extent and time course of the generation of plasma membrane lesions.

Cells at a density of 5 X 10^6/mL in Buffer K were monitored for permeability lesions in the presence of 25 μM ethidium bromide at 25°C. Fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 580 nm in a Perkin Elmer Model MPF-4 fluorescence spectrophotometer. Slit widths were 2 and 12 nm respectively. The positive control consisted of a sample of cell suspension which had been disrupted by sonication.

I. QUIN 2 FLUORESCENCE ASSAY

Quin 2 is a fluorescent tetracarboxylate anion which undergoes about a fivefold increase in fluorescence signal upon the binding of calcium which it chelates with a 1:1 stoichiometry (Tsien et al., 1982, 1984). Owing to its highly charged nature, Quin 2 does not penetrate plasma membranes appreciably. However, esterification of the carboxylate groups to produce Quin 2-acetoxymethyl ester (Quin 2/AM) results in a derivative which, being uncharged and lipid soluble, readily crosses the plasma membrane bilayer. Inside the cell, cytosolic esterases cleave Quin 2/AM back to the lipid insoluble tetracarboxylate compound which is thus trapped in the cell. Quin 2 appears to diffuse throughout the cytosol and nucleus, without penetrating the mitochondria, lysosomes, secretory granules, and endoplasmic reticulum.

Neutrophils were suspended in HBSS, pH 7.4, at a density of 10 X 10^6 cells/mL and incubated for 30 min in the presence or absence of 1 µg/mL pertussis toxin. After 30 min, Quin 2/AM, at a final concentration of 10 µM, was added to each cell suspension and the incubation was continued for another 30 min. At the end of the total 1 hour incubation time, cells were centrifuged and washed once, then resuspended in HBSS at a concentration of 2 X 10^6 cells/mL for fluorescence measurements. The latter were conducted in a Perkin Elmer Model MPF-4 fluorescence spectrophotometer, thermostatically controlled at 37°C. The excitation and emission wavelengths were 339 nm and 492 nm with 5 nm and 15 nm slits respectively. Maximal and minimal fluorescence signals were obtained by lysing the Quin 2-loaded cells with Triton X-100 (1 %) in the presence of either 1.6 mM calcium (F_{max}) or 2 mM EGTA (F_{min}). However, it is worth noting that, at neutral pH, the F_{min} value will be artifactual, as the calcium affinity of EGTA is inadequate to effectively chelate all of the free calcium at pH values less than 8.

Although Quin 2 has achieved prominence as the most suitable probe for the investigation of calcium elevation in the neutrophil, a number of properties of this dye render it unsuitable for quantitative determination of calcium levels (Tsien et al., 1984). The fact that the excitation wavelength, 339 nm, is known to excite autofluorescence from certain endogenous compounds, including reduced pyridine nucleotides, makes baseline readings misleading Furthermore, owing to its high calcium affinity, quin 2 saturates above 1 to 2 μM, concentrations which are in the range of cytoplasmic calcium levels in the activated cell. Quenching of quin 2 fluorescence by endogenous heavy metals is another potential source of artifact (Arsian et al., 1985).

J. SAMPLE PREPARATION PROCEDURE FOR STUDY OF INOSITOL PHOSPHATE PRODUCTION

The sample preparation procedure used in phosphoinositide turnover studies was based on a modification of the procedure of Bradford and Rubin (1985a). Radiolabelling of the phosphoinositides was achieved by incubating neutrophil suspensions for 2 hr at 37°C with 75 to 100 μCi of $[^3H]$myo-inositol (16 Ci/mmol). The neutrophils were suspended at a cell density of 10^8 cells/mL in HBSS which was supplemented with 10% fetal calf serum to minimize cell clumping. In an effort to maximize metabolic activity, incubations were carried out under a 95% O_2/5% CO_2 atmosphere with periodic agitation.

At the end of the incubation, cell suspensions were diluted with HBSS and subjected to two 10 min washes by centrifugation at 400 X g. The neutrophils, upon resuspension at 40 X 10^6 cells/mL in protein- and inositol-free HBSS, were then incubated for varying time intervals at 37°C. Pretreatments with dibutyryl cyclic AMP, pertussis toxin, and PMA were carried out at this time. During the last 10 min of the incubation, 10 mM LiCl was administered, lithium ion being a well-established inhibitor of $InsP_1$ breakdown by inositol 1-phosphomonoesterase (Hallcher and Sherman, 1980). Aliquots of 500 μL (containing approximately 20 X 10^6 cells) were then stimulated by the reagents in question for fixed time intervals. Reaction were terminated by the addition of 500 µL of ice-cold 10% trichloroacetic acid.

Samples were then set on ice for at least 10 min, followed by a 600 X g centrifugation for 5 min at 4°C. The supernatant was retained while the pellet was resuspended in 0.4 mL of 5% trichloroacetic acid, containing 1 mM EDTA. The centrifugation procedure was then repeated, the supernatant retained, and the pellet washed once more, this time with 0.4 mL deionized distilled water. Supernatants from the three centrifugations were then pooled for individual samples and washed with a fourfold excess of diethylether, to remove organic contaminants. Each sample was subsequently adjusted to pH 7 to 9 by the dropwise addition of 0.5 M KOH. Samples were either subjected to chromatographic resolution of the inositol phosphates immediately or else stored for 12 to 24 hour periods at -70°C.

K. CHROMATOGRAPHIC SEPARATION OF INOSITOL PHOSPHATES

Inositol phosphates were separated by anion exchange chromatography as described by Berridge (1983). AG1 X8 200-400 mesh (BioRad, formate form) was suspended in 0.1 M formate supplemented with 5 mM inositol. The suspended resin was added to disposable 15 mL polypropylene columns (Econocolumn, BioRad) in 0.3 to 0.5 mL volumes. To eliminate non-specific binding, the resin was washed initially with 1 mL of the suspension buffer prior to application of the sample. Upon draining of the samples to the top of the resin bed, the columns were eluted with 10 mL of deionized water in order to remove labelled inositol. This was followed by elution with 8 mL volumes of a series of formate buffers. Buffer 1, containing 5 mM sodium borate and 60 mM sodium formate, eluted glycerylphosphatidylinositol. Buffer 2, containing 0.1 M formic acid and 0.2 M ammonium formate, eluted inositol 1-phosphate. Inositol 1,4-bisphosphate was eluted by buffer 3 which contained 0.1 M formic acid and 0.4 M ammonium formate. Buffer 4, containing 0.1 M formic acid and 1 M ammonium formate, eluted inositol 1,4,5-trisphosphate.

Collected fractions were counted in 80% ACS II liquid scintillant in a Beckman LS 6800 liquid scintillation counter. Counts per minute were converted to disintegrations per minute (dpm) by a Beckman program based on a quench curve generated for a group of commercially supplied tritium standards. Radioactive sample peaks were identified by comparison with the peaks obtained using commercially prepared standards for [^{3}H]inositol, [^{3}H]InsP$_1$, [^{3}H]InsP$_2$, and [^{3}H]InsP$_3$. Results were expressed as the percent increase in [^{3}H]inositol phosphate levels in stimulated, as compared to unstimulated, cells.

L. PROTEIN KINASE C ASSAY

Following isolation, neutrophils were resuspended at a density of 20 X 10^6 cells/mL in Tris buffer containing 5 mM EDTA, 0.25 M sucrose, 10 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride (a non-specific protease inhibitor), and 0.1 mM leupeptin (a serine-specific protease inhibitor), pH 7.4 and preincubated for 30 min at 37°C. The latter ingredient was necessary to prevent conversion of protein kinase C to protein kinase M by the serine-specific protease, calpain (Melloni et al., 1985). Following the appropriate stimulation conditions, 1 mL reaction mixtures were terminated by sonication of the cell suspensions by six 10 sec bursts (70 Watts) from a Braunsonic 1510 sonicator. Cell fractionation was achieved by a 60 min centrifugation at 100,000 X g. The supernatant was retained as the cytosolic fraction. The pellet, representative of the membrane fraction, was resuspended in the Tris buffer supplemented with 0.2 % Triton X-100 to achieve solubilization of protein kinase C activity.

The protein kinase C assay was performed according to the procedure of Parente et al. (1986). The standard assay buffer consisted of 25 mM Tris (pH 7.4), 5 mM $MgCl_2$, 0.1 mM ATP, 0.1 M leupeptin, 1 mM $CaCl_2$, 100 nM PMA, 0.625 mg/ml Type II-A histone, 62.5 µg/ml phosphatidylserine, and 10 µM $[\gamma\text{-}^{32}P]$ATP. Protein kinase C activity was determined on the basis of histone phosphorylation occurring upon the addition of 0.2 mL of assay buffer to 50 µL of the cytosolic or membrane fraction. Non-protein kinase C-mediated phosphorylation was quantified in controls using a modified assay buffer containing 25 mM EDTA from which calcium, phosphatidylserine, and PMA were omitted.

Termination of phosphorylation reactions after 15 min was achieved by the simultaneous addition of 15% trichloroacetic acid and 0.8% bovine serum albumin, as the carrier. The precipitate was collected on 2.5 cm glass microfibre filters and counted in scintillated toluene in a Beckman LS 6800 liquid scintillation counter. Results were expressed as the percent increase and decrease in protein kinase C activity in the membrane and cytosolic fractions, respectively.

M. STATICTICAL ANALYSIS OF DATA

Most data points have been expressed as the mean ± standard error of the mean (SEM). Student's unpaired "t" test was used to assess the significance of differences between means. At $p<0.05$, difference was considered significant.

CHAPTER III

RESULTS

RESULTS

A. EFFECT OF THE CALCIUM IONOPHORE, A23187, ON SUPEROXIDE GENERATION IN PHORBOL ESTER-STIMULATED HUMAN NEUTROPHILS

Oxygen radical release from neutrophils stimulated with PMA and A23187 was investigated and quantified with respect to lag period, superoxide generation rate, and total superoxide production.

Superoxide production in response to PMA, over a concentration range from 0.5 nM to 1 µM, is shown in Figure 4. The superoxide production response at all concentrations tended to converge at the same plateau level, a feature indicative of cytochrome c levels becoming limiting. For 1 nM PMA alone, the average lag period was 17.0 ± 6.5 (SE) min. A23187, at concentrations below 5 µM, failed to elicit any superoxide production when used alone. A transient respiratory burst, with a lag period of 4.0 ± 0.2 (SE) min, was observed with 10 µM A23187. Simultaneous stimulation with A23187, over the concentration range of 10^{-8} to 10^{-5} M, decreased the lag interval preceding PMA-induced superoxide production in a dose-dependent manner (Figure 5).

The average superoxide production rate for 1 nM PMA alone was 0.97 ± 0.67 (SE) nmol/min/10^6 cells. For 10 µM A23187, the rate averaged 2.2 ± 0.3 (SE) nmol/min/10^6 cells. Simultaneous addition of A23187 and PMA had a biphasic effect on the superoxide generation rate (Figure 6). At a fixed PMA concentration of 1 nM, the presence of A23187 increased the superoxide generation rate in a dose-dependent manner, with maximal

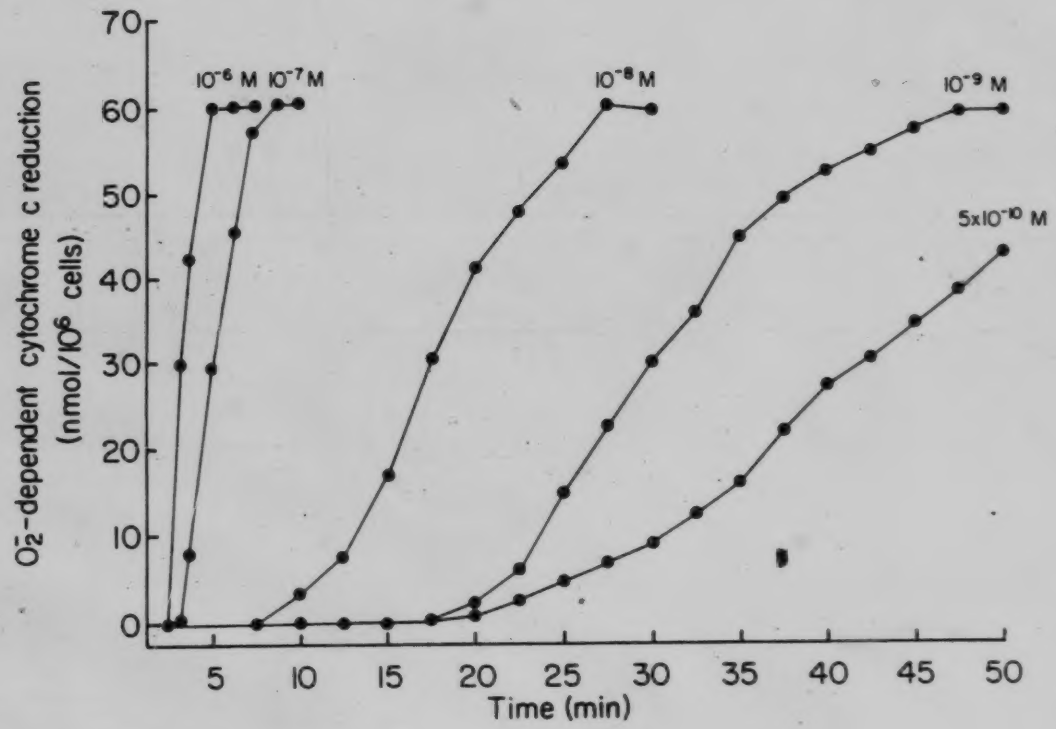


Figure 4: Time course and dose-dependency of PMA-activated superoxide production in the neutrophil. Superoxide production was monitored by cytochrome c reduction, as described in Methods. The results shown were obtained from a single experiment which is representative of at least 10 experiments.

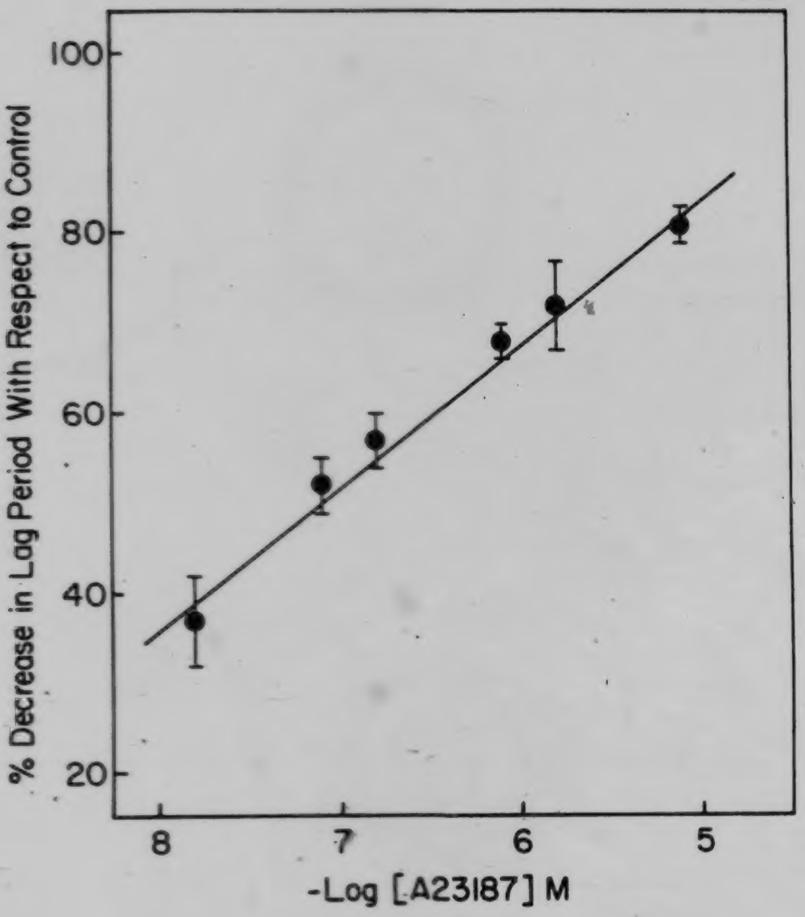


Figure 5: Effect of the simultaneous addition of A23187 and 1 nM PMA on the lag period preceding superoxide generation in a 1 X 10^6 cell/mL suspension of neutrophils. For 1 nM PMA alone, the lag period was 17.0 ± 6.5 min. Each point represents the mean ± SEM of at least five determinations.

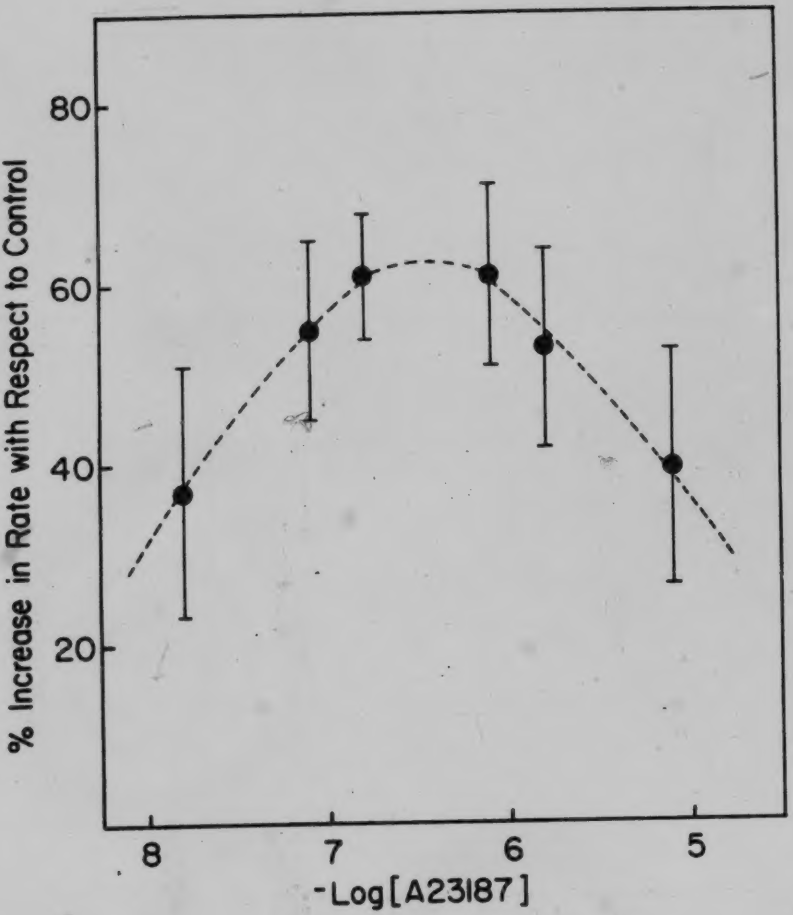


Figure 6: Effect of the simultaneous addition of A23187 on the rate of superoxide production in a 1 X 10^6 cell/mL suspension of neutrophils stimulated with 1 nM PMA. For 1 nM PMA alone, the average rate of superoxide production was 2.2 ± 0.3 nmol/min/10^6 cells. Each point represents the mean ± SEM of at least five different experiments.

potentiation occurring over the A23187 concentration range between 0.1 and 1 μM. At higher A23187 concentrations, the rate of PMA-induced superoxide generation was reduced. At 10 μM concentrations of the ionophore, the absorbance tracing had a biphasic aspect due to an initial transient burst accounted for by the ionophore. Rate was calculated from the linear portion of the second phase of the curve at which the effects of the phorbol ester were expressed.

As PMA activation of 1 X 10^6 cell/mL incubation mixtures resulted in spurious plateaus due to limiting concentrations of cytochrome c, estimates of total superoxide production were based on experiments in which cell densities were reduced to 2.5 X 10^5 cells/mL. Total superoxide production for 1 nM PMA without A23187 averaged 59.4 ± 16.2 (SE) nmol/10^6 cells, while 10 μM A23187 produced only 5.4 ± 1.1 (SE) nmol/10^6 cells. The A23187 response generally reached a plateau in about 10 min, while the PMA response often continued for 50-80 min before attaining a plateau level. Although A23187, at concentrations below 0.5 μM had no effect on total PMA-induced superoxide production, higher concentrations brought about a marked reduction in the overall quantity of superoxide generated (Figure 7). The ID_{50} of this inhibitory effect was estimated to be 1.3 μM.

The calcium dependence of these stimulatory and inhibitory effects was demonstrated by a comparison of overall superoxide production in cell suspensions exposed to PMA and A23187 in Ca^{2+}-free and Ca^{2+}-enriched (1.6 mM) extracellular media (Table 1). In agreement with previous reports (Lehmeyer et al., 1979), superoxide generation by PMA-activated cells was not affected by the absence of extracellular Ca^{2+}.

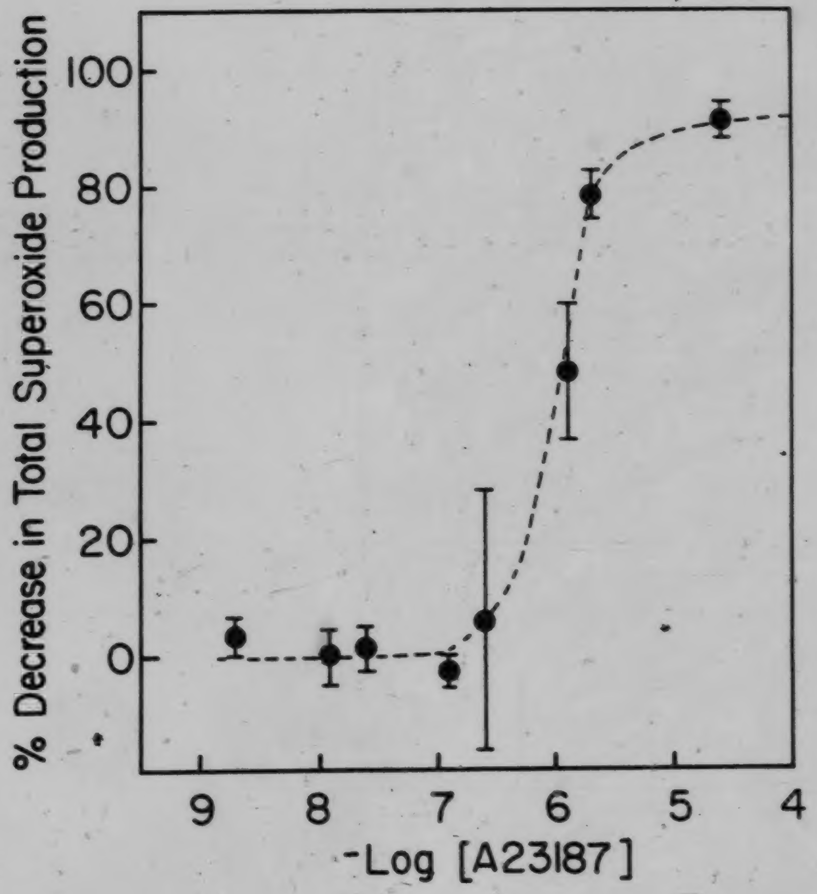


Figure 7: Effect of the simultaneous addition of A23187 and 1 nM PMA on total superoxide production in a 2.5 X 10^5 cell/mL suspension of neutrophils. Each point represents the mean ± SEM for at least three different experiments.

Table 1: Effect of extracellular calcium on inhibition of superoxide release by A23187

Condition	Superoxide released (nmol/10^6 cells)
1 nM PMA (+ Ca^{2+})	59.4 ± 16.2 (n.s.)*
1 nM PMA (- Ca^{2+})	57.0 ± 10.3 (n.s.)
1 nM PMA + 1 μM A23187 (+ Ca^{2+})	26.9 ± 5.7 ($p<0.05$)
1 nM PMA + 1 μM A23187 (- Ca^{2+})	60.1 ± 13.7 (n.s.)

*Each value represents the mean ± SEM of at least three experiments.

However, neither inhibitory nor synergistic effects were observed in the presence of A23187 in the Ca^{2+}-free medium, thus eliminating the possibility that the ionophore modulates neutrophil functions through disruption of membrane structure or activation of enzymes by calcium-independent processes.

Attempts were also made to characterize the interactive effects of A23187 and PMA on the lysozyme release response. Synergistic interactions between protein kinase C activation and calcium mobilization were evident with respect to the release of this enzyme, in confirmation of the work of Kajikawa et al. (1983) and White et al. (1984). As degranulation appeared to be less sensitive to phorbol ester effects than was superoxide production, a 250 nM concentration of PMA was used for this part of the study. Synergism was pronounced only during the first few minutes of exposure to the stimuli (Figure 8). At the five minute time point, 0.1 µM A23187 or 250 nM PMA alone caused less than 5% release of lysosomal enzyme stores, while administration of the two stimuli in combination resulted in the release of over 25% of granular lysozyme. At 10 minutes, the response was merely additive, while at later time points the massive Ca^{2+}-dependent release evoked by the ionophore masked the phorbol ester-induced contribution.

In an attempt to determine whether high A23187 concentrations exerted inhibitory effects on the degranulation, as well as the superoxide production, component of the inflammatory response, lysozyme release was assayed following a fifteen minute exposure to 250 nM PMA and 10 µM A23187, alone and in combination. PMA alone caused the release of 22 ± 3 (SE) % of lysozyme stores under these conditions,

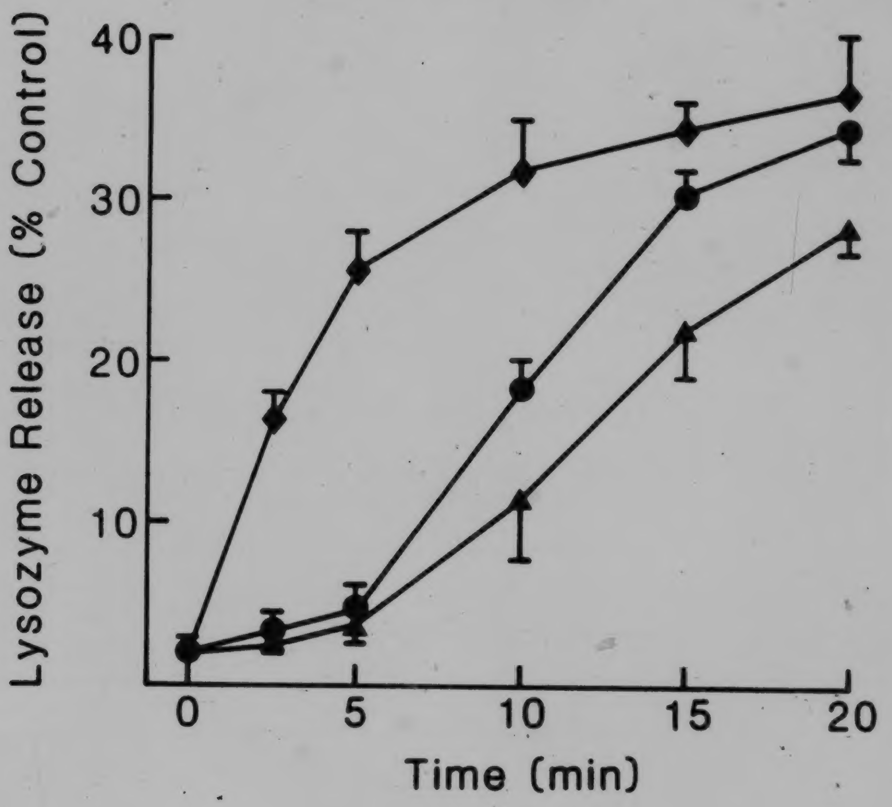


Figure 8: Effect of PMA and A23187 on lysosomal enzyme release. Lysozyme release was assayed following exposure of neutrophils to 2.5 X 10^{-7} M PMA (▲) and 1 X 10^{-7} M A23187 (●), alone and in combination (◆). Lysozyme release was assayed spectrophotometrically by the reduction in absorbance of a suspension of M. lysodeikticus, as described in Methods. The results shown represent the mean ± SEM of at least 7 determinations. At the 2.5 and 5 min time points, the response obtained in the presence of both A23187 and PMA was significantly greater than the sum obtained when the two stimuli were administered separately ($p<0.05$).

while A23187 caused 40 ± 6 (SE) % release. Notably, the response to 10 μM A23187 exhibited no appreciable lag and reached a plateau in about 5 min. A23187 and PMA in combination at these concentrations were found to result in the release of 42 ± 4 (SE) % of lysozyme stores. Even when earlier points in the time course were addressed, the combined effects of the two stimuli did not appear to exceed the effect achieved with A23187 alone (data not shown). The fact that the combined effect was less than additive may represent inhibition of the PMA-induced component of the response at high concentrations of the ionophore. If this were the case, it would suggest that the inhibitory effects of A23187 are exerted at an intermediate stage in the transduction pathway, perhaps at the level of protein kinase C or at steps closely following protein kinase C activation. However, an alternative, and doubtless more probable, explanation is that 10 μM A23187, itself, elicited a maximal release response from cells such that potentiation of PMA effects could not be resolved (ie. the fraction of granular stores available for PMA-induced release was released by A23187). This interpretation, if correct, would imply that A23187 is specifically toxic for the superoxide generation component of the inflammatory response.

Elevated A23187 concentrations appeared not be cytotoxic to neutrophils by the criterion of lactic dehydrogenase release (Figure 9). The release of this cytosolic enzyme never exceeded the basal level of <5%, even with supramicromolar concentrations of the ionophore. As inhibitory effects were not observed in the cell-free xanthine oxidase reductase system, A23187 did not appear to affect the cytochrome c reduction method by reoxidizing reduced cytochrome c or scavenging superoxide radicals.

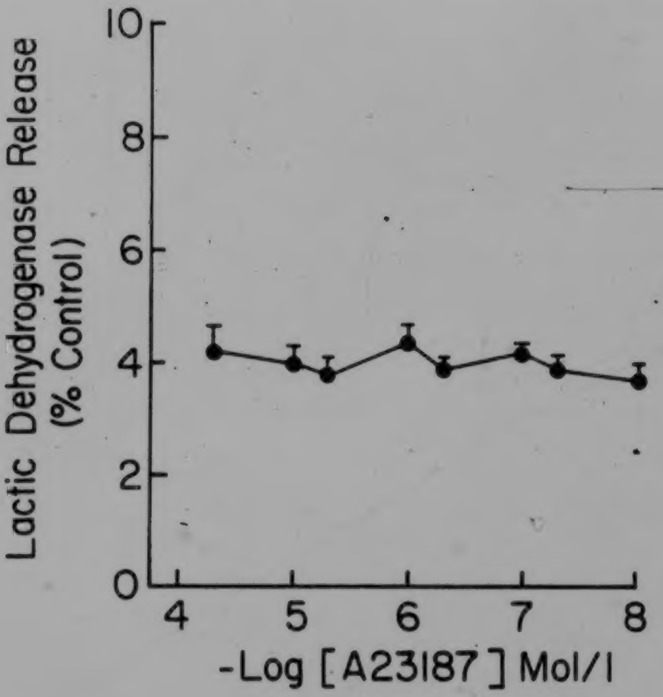


Figure 9: Lactic dehydrogenase release from neutrophils, following a 10 min incubation with varying concentrations of A23187. Lactic dehydrogenase release from untreated cells averaged 4.0 ± 0.3% of the total cellular content of lactic dehydrogenase. Results shown represent the mean ± SEM for at least 3 experiments.

B. PHORBOL ESTER EFFECTS ON CHEMOATTRACTANT- AND PROSTAGLANDIN-INDUCED CYCLIC AMP PRODUCTION

Fmet-leu-phe-induced superoxide production was inhibited by elevation of intracellular cyclic AMP levels through simultaneous addition of prostaglandin E_1, a 90 min preincubation with cholera toxin, a 10 min preincubation with forskolin, or a 30 min preincubation with dibutyryl cyclic AMP (Table 2). Both the rate and total amount of superoxide production were markedly reduced by the elevation of cAMP levels under these circumstances. Conversely, the superoxide production response elicited by 10 nM PMA was unaffected by manipulation of intracellular cAMP levels through use of these agents.

A cyclic AMP radioimmunoassay was used to quantitate cAMP production in response to fmet-leu-phe and to prostaglandin E_1. These agents were selected because, owing to their action at the level of cell surface receptors, elevated cAMP levels can be achieved in the absence of an appreciable lag period. Fmet-leu-phe, at a concentration of 1 μM, was found to elict a detectable elevation in cAMP levels within 15 sec (Figure 10). The maximal response, represented by a 2 fold elevation of cyclic AMP levels, occurred within 30 sec of addition of the stimulus, after which there was a progressive decline to basal levels between 1 and 3 min. The elevation in cAMP levels induced by prostaglandin E_1 was also evident within 15 sec (Figure 11). However, this response was of greater magnitude, achieving a 3 fold elevation over basal cyclic AMP levels which peaked at 1 min and remained elevated over 5 min, after which time a gradual decline in the direction of basal levels was noted.

Table 2: Effect of cyclic AMP and adenylate cyclase agonists on fmet-leu-phe- and PMA-induced superoxide production.

	fmet-leu-phe (10^{-6} M) (nmol/10^6 cells)	PMA (10^{-8} M) (nmol/10^6 cells)
no addition	21 ± 4.0	60 ± 12*
prostaglandin E_1 (1 μM)	3 ± 0.6 ($p<0.05$)	59 ± 9 (n.s.)
cholera toxin (1 μg/mL)	7 ± 0.6 ($p<0.05$)	61 ± 8 (n.s.)
forskolin (0.1 mM)	6 ± 1.1 ($p<0.05$)	59 ± 7 (n.s.)
dibutyryl cAMP (1 mM)	4 ± 0.8 ($p<0.05$)	62 ± 10 (n.s.)

*Each value represents the mean ± SEM of at least five different experiments.

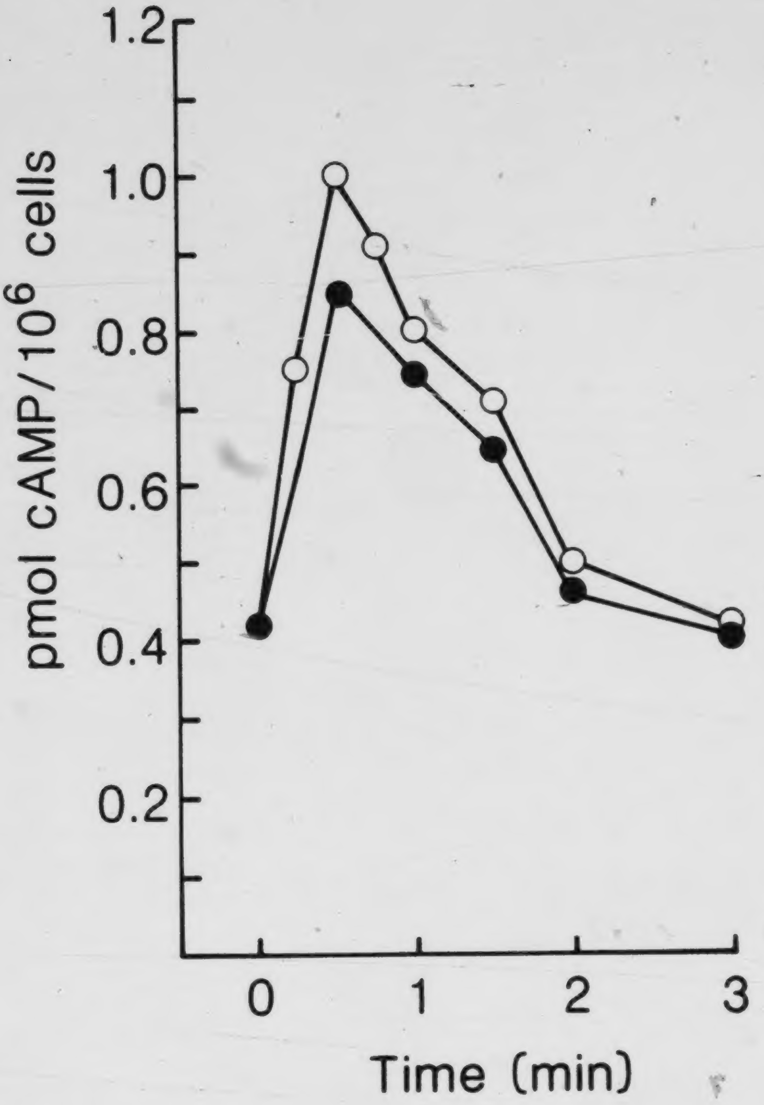


Figure 10: Effect of 1 μM fmet-leu-phe on cAMP levels in the neutrophil in the presence (○) or absence (●) of a 10 min preincubation with 0.1 μM PMA. Cyclic AMP levels were determined by radioimmunoassay, as described in Methods. The values shown were obtained from a single experiment which is representative of at least 5 experiments.

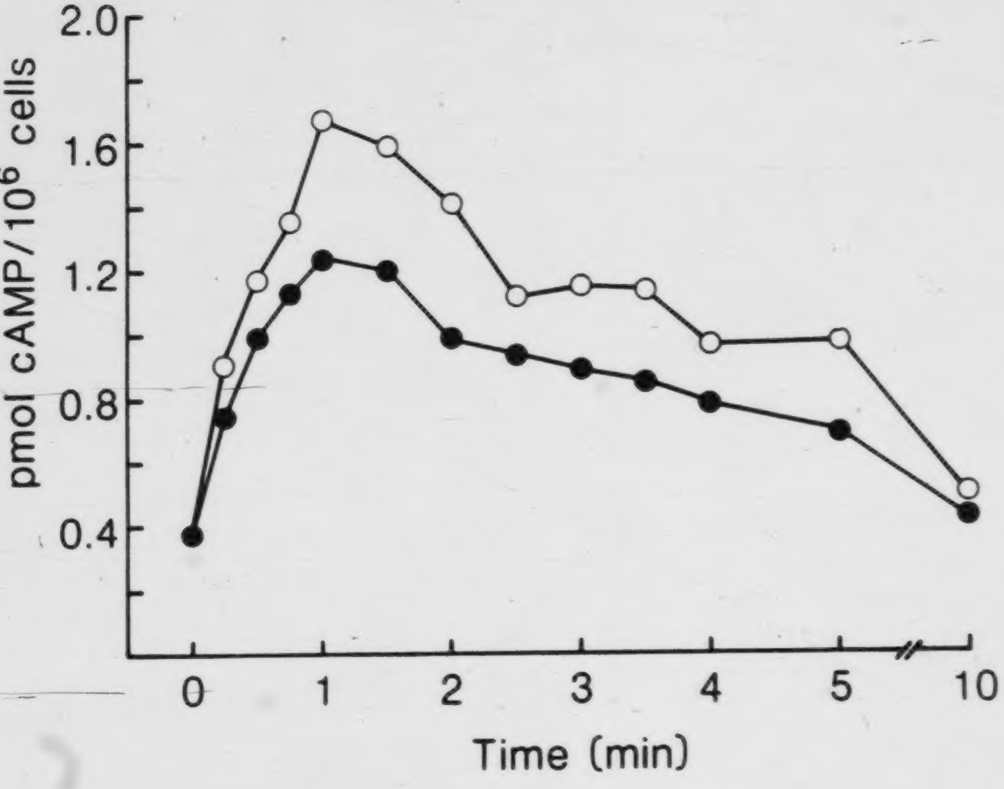


Figure 11: Effect of 1 μM PGE_1 on cAMP levels in the neutrophil in the presence (○) or absence (●) of a 10 min preincubation with 0.1 μM PMA. The values shown were obtained from a single experiment which is representative of at least 7 experiments.

PMA pretreatment, in itself, had no effect on basal cAMP levels in unstimulated cells (Figure 12). A 10 min preincubation with 0.1 µM PMA, prior to addition of fmet-leu-phe or PGE_1, was not found to inhibit the cyclic AMP responses induced by either of these stimuli. In fact, both fmet-leu-phe (Figure 10) and PGE_1 (Figure 11) cAMP responses were mildly enhanced by phorbol ester pretreatment, fmet-leu-phe by 20 ± 5% (N=5) and PGE_1 by 38 ± 9% (N=7) at their peak levels. The time course of the cAMP responses were, however, parallel in phorbol ester-treated and untreated cells. Notably, this phorbol ester-induced potentiation could not be consistently detected at lower concentrations of the tumor promotor.

C. EFFECT OF GUANINE NUCLEOTIDE ANALOGUES ON LYSOZYME SECRETION FROM SAPONIN-PERMEABILIZED NEUTROPHILS

Neutrophil permeabilization in the presence of saponin was monitored by the increase in ethidium bromide fluorescence signal monitored over time in a 5 X 10^6 cell/mL suspension (Figure 13). Saponin, when present at 0.005%, resulted in a gradual increase in cell permeability which was half-maximal at about 8 min. That sub-maximal permeabilization was not associated with irreversible cytotoxity was demonstrated by the fact that cells washed in saponin-free Buffer K and resuspended in HBSS regained the ability to exclude ethidium bromide, as well as Trypan blue. Although, the ethidium bromide signal for permeabilized and 'resealed' cells was somewhat higher than the resting level for cells which had not been subjected to permeabilization, this level was stable and did not increase with time. Furthermore, cells

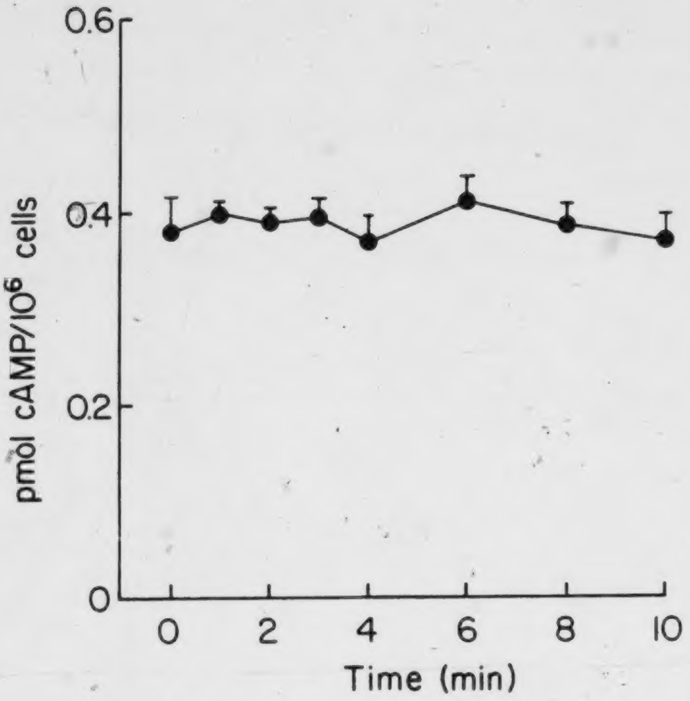


Figure 12: Effect of 0.1 μM PMA on cAMP levels in the neutrophil. The values shown represent the mean ± SEM of at least 3 experiments.

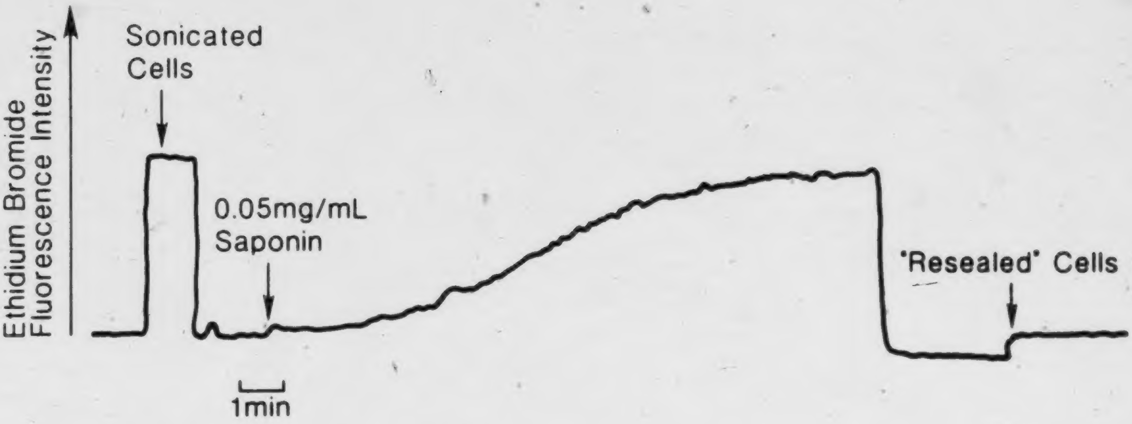


Figure 13: Permeabilization of a 5 X 10^6 cell/mL suspension of neutrophils in the presence of 0.005% saponin. Permeabilization was monitored by the increase in fluorescence of the nuclear stain, ethidium bromide, as described in Methods. A sonicated cell suspension served as the positive control. "Resealed cells" consisted of a sample of cell suspension which had been exposed to 0.005% saponin for 7 min, then washed repeatedly in detergent-free buffer. The tracings shown depict the results obtained from a single experiment which is representative of at least five experiments.

permeabilized and 'resealed' in this manner retained their capacity to respond with a respiratory burst in the presence of fmet-leu-phe, albeit at a reduced level.

Cell permeabilization was also monitored by assaying for release of the cytosolic enzyme, lactic dehydrogenase, at fixed time intervals over a 15 min exposure to 0.005% saponin (Figure 14). Although a general trend was evident, cell samples obtained from different donors showed a great deal of variability in their ability to withstand exposure to saponin. For this reason, the duration of the permeabilization procedure often had to be individualized for different cell preparations. Beyond the 10 min time point, lactic dehydrogenase loss generally exceeded 50% of the total cellular supply, a situation likely to be associated with a considerable amount of cytotoxicity.

In subsequent studies a 5 to 7 min permeabilization was performed in the presence of various nucleotides and their analogues. Following repeated washings in detergent-free permeabilization buffer, cells were resuspended in either normal or calcium-free HBSS. After fixed time intervals of incubation, cells were sedimented and assayed for lysozyme and lactic dehydrogenase release. Cytochalasin B was not included in these studies, as cells which been permeabilized tended to release granular stores into the extracellular medium quite readily (Smolen and Stoehr, 1985). Whereas lysozyme is a granular enzyme released in response to cell activation, lactic dehydrogenase is a cytosolic enzyme, the loss of which can be considered a measure of cytotoxicity.

Control cells resuspended in normal HBSS lost approximately 15% of their granular enzyme stores over a 16 min time interval, presumably by

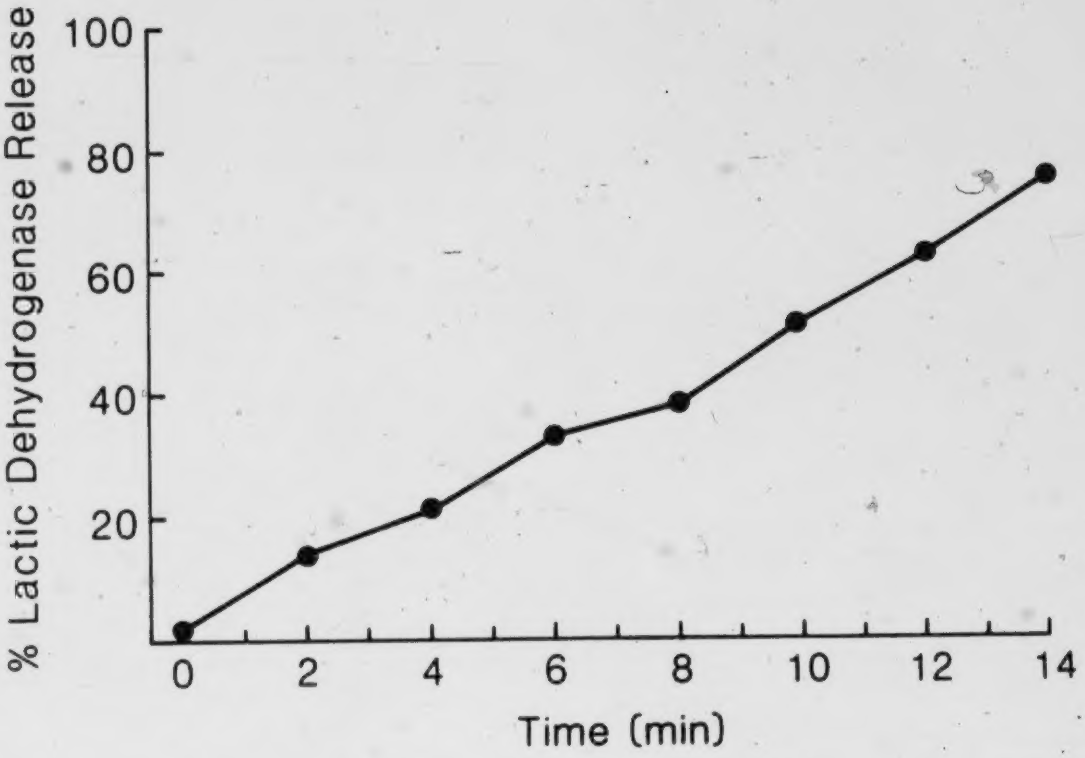


Figure 14: Permeabilization of a 10 X 10^6 cell/mL neutrophil suspension, as monitored by release of the cytosolic enzyme, lactic dehydrogenase. The values shown represent the means of duplicate determinations from a single experiment which is representative of at least 3 experiments.

means of calcium leakage through incompletely sealed lesions (Figure 15). In cells resuspended in calcium-free HBSS, basal lysozyme loss was only about 5%. Cells loaded with ATP, ADP, GTP, GDP, and cGMP showed no consistent change in lysozyme release over that observed in control cells which had been permeabilized in the absence of nucleotides (Figure 16). However, cells permeabilized in the presence of the non-hydrolyzable guanine nucleotide-analogues, Gpp(NH)p and GTPγ-S, displayed an enhancement of lysozyme release in the presence of calcium-containing, but not calcium-free, HBSS (Figure 17). Enhancement reached approximately 40% at optimal concentrations of both analogues. Cells from all suspensions, regardless of treatment, showed a small and constant degree of lactic dehydrogenase release under these circumstances, which did not generally exceed 10% of the lactic dehydrogenase content of resealed cells (Figure 18).

D. FLUORIDE-ACTIVATED SUPEROXIDE RADICAL PRODUCTION IN HUMAN NEUTROPHILS

Upon exposure to millimolar concentration of NaF, human neutrophils responded by the generation of superoxide radicals, as monitored by the reduction of ferricytochrome c (Figure 19). As reported previously (Curnutte et al., 1979; Wong, 1983), fluoride-induced superoxide production was characterized by a lag period of 4 to 20 min. This delay distinguished the fluoride response from that evoked by fmet-leu-phe which had a rapid onset of 15 sec following addition of the chemoattractant. Furthermore, whereas fluoride elicited a sustained response of 30 to 60 min duration, the fmet-leu-phe response tended to plateau abruptly after about 2 min.

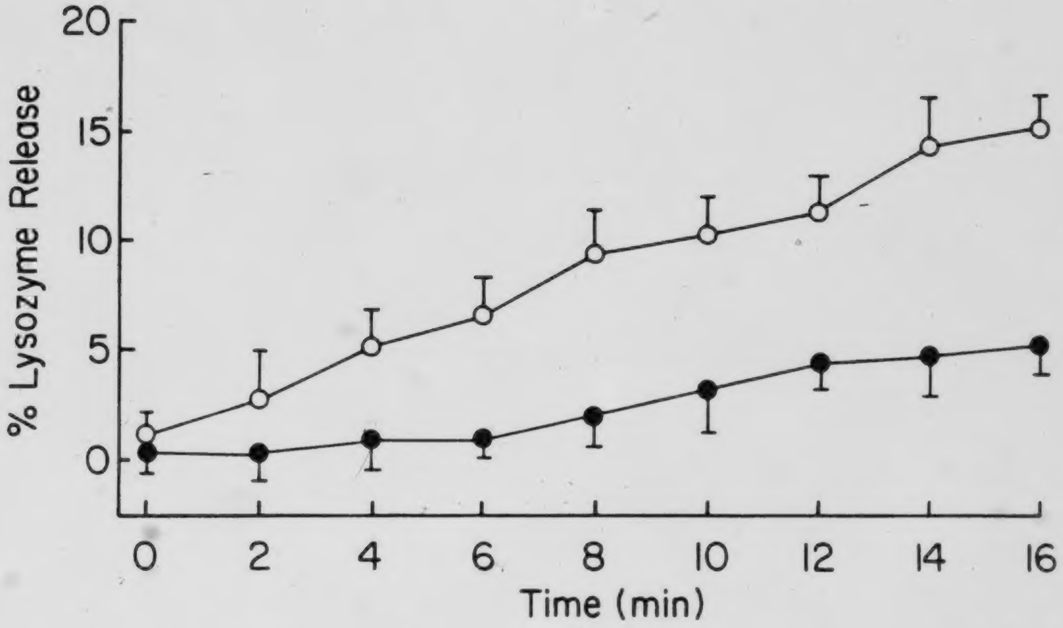


Figure 15: Basal lysozyme loss from permeabilized and 'resealed' 10 X 10^6 cell/mL suspensions in 1.6 mM Ca^{2+} (○) and Ca^{2+}-free HBSS (●). Values shown represent the mean ± SEM of at least 5 experiments.

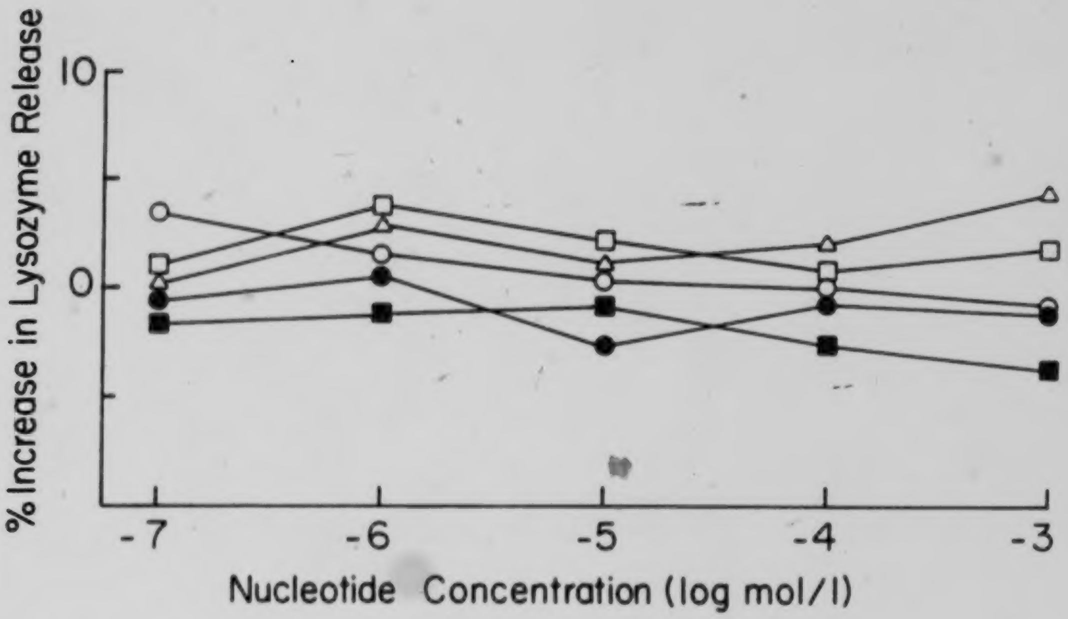


Figure 16: Lysozyme release from 'resealed' cell suspensions which had been permeabilized in the presence of the nucleotides GTP (△), ATP (□), cGMP (○), GDP (●), and ADP (■), then resuspended in normal HBSS for 7 min. Lysozyme release from control cells, which had been permeabilized in the absence of nucleotides, was typically in the range of 10% of the total lysozyme stores. Error bars have been excluded for the sake of clarity, but were typically in the range of ± 3.5%.

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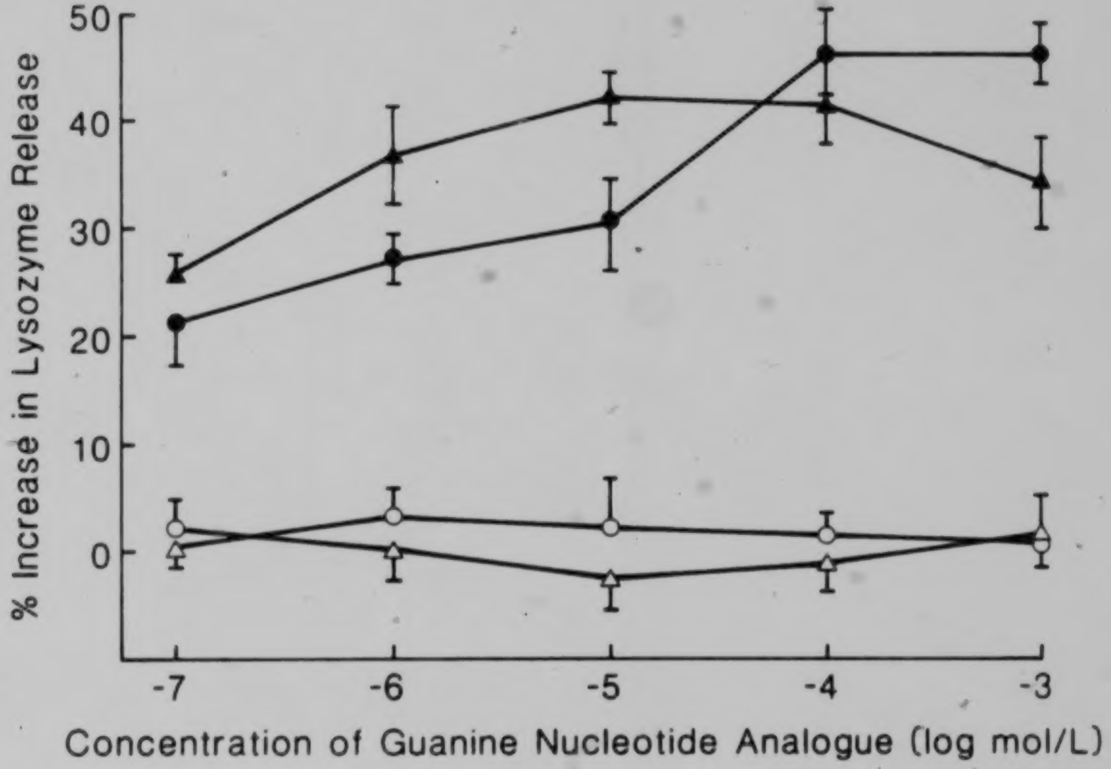


Figure 17: Effect of non-hydrolyzable guanine nucleotide analogues on lysozyme release. Lysozyme activity was assayed in the supernatants obtained from 'resealed' 10 X 10^6 cell/mL suspensions which had been permeabilized in the presence of the guanine nucleotide analogues, Gpp(NH)p (○ ●) or GTPγ-S (△ ▲), then resuspended in normal (1.6 mM Ca^{2+}) HBSS (closed symbols) or Ca^{2+}-free HBSS (open symbols) for 7 min. Typical basal values for lysozyme release in the absence of guanine nucleotide analogues were in the range of 10% in the presence of Ca^{2+} and 3% in the absence of Ca^{2+}. Values shown represent the mean ± SEM of at least 8 experiments.

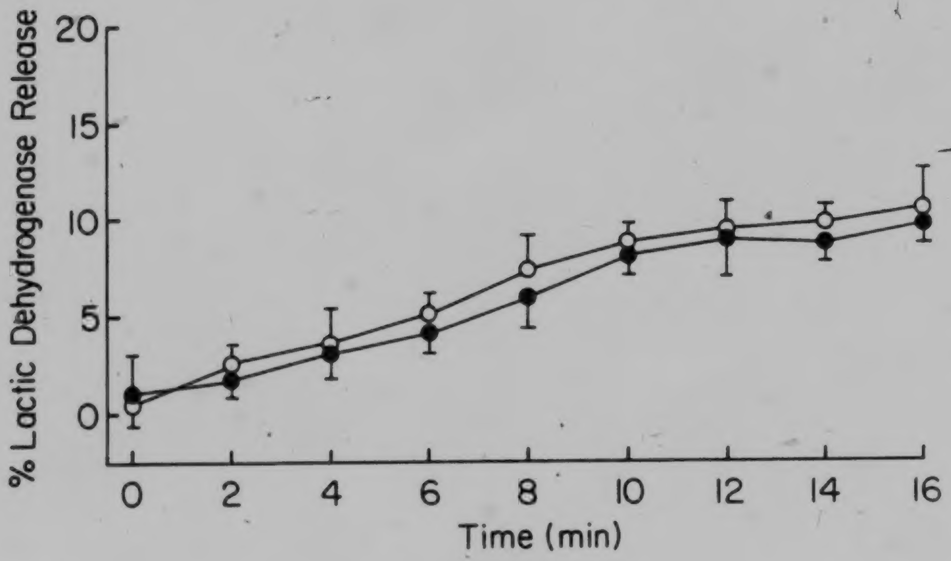


Figure 18: Effect of GTPγ-S on the release of lactic dehydrogenase. Lactic dehydrogenase loss from permeabilized and 'resealed' 10 X 10^6 cell/mL suspensions was monitored in control (○) groups and cells which had been loaded with 1 mM GTPγ-S (●). Values shown represent the mean ± SEM of at least 3 experiments.

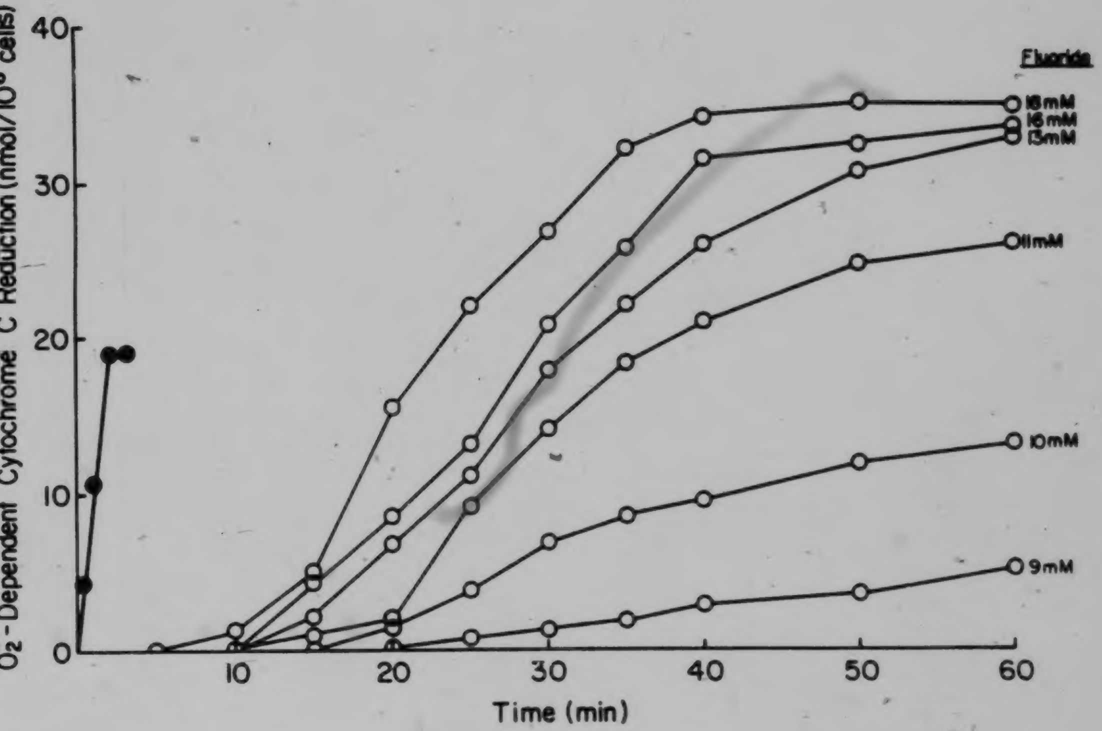


Figure 19: Time course and dose-dependency of superoxide production in neutrophils in the presence of varying concentrations of NaF (○) and 10^{-6} M fmet-leu-phe (●). Superoxide production was monitored by cytochrome c reduction as described in Methods. The results shown were obtained from a single experiment which is representative of at least 10 experiments.

The usual threshold concentration for the fluoride-induced respiratory burst was 9 to 10 mM. Reduction in the lag period and acceleration of the rate were observed to be dose-dependent up to an optimal concentration of 18 mM, above which autoinhibitory effects became manifest. To exclude the possibility that alterations in tonicity might be a contributing factor in the response, studies were performed using modified HBSS in which the NaCl concentration was reduced such that the final salt concentration, following the addition of NaF, would be isotonic. Equal concentrations of NaCl were added to unstimulated controls. These manipulations were not found to affect the kinetics of fluoride-induced superoxide production (Table 3). It is worth noting that, owing to fluoride's ability to complex with calcium and magnesium ions in the medium, actual fluoride ion concentrations in the reaction mixtures may be somewhat lower than anticipated. No obvious precipitates were observed in the course of experiments, however.

As shown in Table 4, a 30 min preincubation with dibutyryl cAMP reduces the fmet-leu-phe superoxide response by approximately 80%. Likewise, fmet-leu-phe-induced superoxide generation is almost wholly abolished by a 90 min preincubation with 1 µg/mL pertussis toxin. Figure 20 shows the time course of this inhibitory effect. Fluoride-induced superoxide production differed from that elicited by the chemotactic peptide in being resistant to the inhibitory effects of pertussis toxin or elevated intracellular cAMP levels (Table 4). However, the fluoride-activated respiratory burst was dependent on the presence of extracellular calcium, as indicated by the reduction in both

Table 3: Kinetic parameters of the superoxide production response for 18 mM sodium fluoride in normal and modified HBSS

	Normal HBSS	Modified HBSS
lag (min)	8.0 ± 4.0	7.0 ± 3.5 (n.s.)*
rate (nmol/min)	2.0 ± 0.6	2.2 ± 0.4 (n.s.)
total O_2^- (nmol/10^6)	38 ± 5	37 ± 6 (n.s.)

*Values shown represent the mean ± SEM of at least 3 experiments.

Table 4: Effect of pertussis toxin and dibutyryl cAMP preincubation on total superoxide production by fmet-leu-phe- and fluoride-stimulated neutrophils.

	fmet-leu-phe (1 µM)	NaF (18 mM)
no addition	21 ± 6	38 ± 5*
1 µg/mL pertussis toxin (90 min)	2 ± 0.9 ($p<0.05$)	36 ± 6 (n.s.)
1 mM dibutyryl cAMP (30 min)	5 ± 1 ($p<0.05$)	37 ± 2 (n.s.)

*Each value represents the mean ± SEM from at least five experiments.

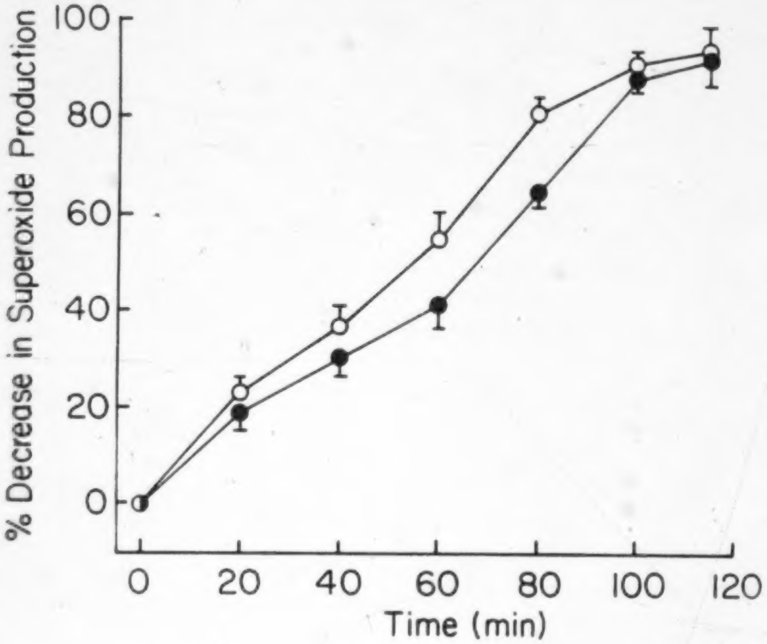


Figure 20: Time course for pertussis toxin inhibition of fmet-leu-phe-evoked superoxide production in human neutrophils. Neutrophil suspensions at a density of 10 X 10^6 cells/mL were incubated with 1 µg/mL (○) or 0.5 µg/mL (●) pertussis toxin for the indicated time intervals, then assayed for superoxide production upon exposure to 1 µM fmet-leu-phe. The results are expressed as the percent decrease in superoxide production in pertussis toxin-treated cells, as compared to control cells at each time point indicated. The values shown are the mean ± SEM for at least five determinations.

the rate and total amount of superoxide produced in a calcium-free modified HBSS, containing 1 mM EGTA (Table 5).

Aluminum (Al^{3+}) contaminants present in solutions stored in common glassware are believed to represent a necessary cofactor for fluoride's action on G-proteins (Sternweis and Gilman, 1982). As one report suggests that fluoride responses can be enhanced by additional aluminum supplementation of solutions (Blackmore et al., 1985), cytochrome c assays were performed in the presence of added $AlCl_3$. In my studies, addition of micromolar amounts of $AlCl_3$ to incubation mixtures had no potentiating effect upon cellular responses, even at submaximal fluoride concentrations, and, in fact, resulted in inhibitory effects (Table 6). Inhibition was concentration dependent for aluminum in the 1 to 100 µM range and influenced lag period, rate, and the total amount of superoxide generated. At 100 µM aluminum, total superoxide production was suppressed by 78%. For this reason, supplementation of assay mixtures with aluminum salts was not indicated. Interestingly, these inhibitory effects were specific to the fluoride-induced respiratory burst, as the fmet-leu-phe-induced response was unaffected, even at the highest aluminum concentration used (results not shown).

E. FLUORIDE-INDUCED ELEVATION OF CYTOSOLIC CALCIUM CONCENTRATIONS IN HUMAN NEUTROPHILS

Upon exposure to millimolar concentrations of NaF, human neutrophils respond with an increase in cytosolic free calcium levels $(Ca^{2+})_i$, as monitored through use of the fluorescent calcium probe, Quin 2 (Figure 21). The fluoride-induced increase in cytosolic calcium

Table 5: Effect of extracellular calcium on the kinetic parameters of superoxide production induced by 18 mM NaF.

	+Calcium	-Calcium
lag (min)	8 ± 2.1	8 ± 2.3 (n.s.)*
rate (nmol/min)	2.9 ± 0.4	1.1 ± 0.2 ($p<0.05$)
total O_2^- (nmol/10^6 cells)	38 ± 3	16 ± 4 ($p<0.05$)

*Values shown represent the mean ± SEM of at least five experiments.

Table 6: Effect of Added $AlCl_3$ on O_2^- production in the presence of 15 mM NaF

$[AlCl_3]$ (μM)	Lag (min)	Rate (nmol/min)	Total O_2^- Production (nmol/10^6 cells)
0	11.3 ± 1.0	1.1 ± 0.2	36.8 ± 5.6*
1	13.3 ± 1.1	0.9 ± 0.1	29.8 ± 4.7
10	13.8 ± 1.0	0.8 ± 0.1	29.2 ± 4.7
100	26.2 ± 4.0	0.3 ± 0.2	8.1 ± 2.1

*Values shown represent the mean ± SEM of at least four experiments.

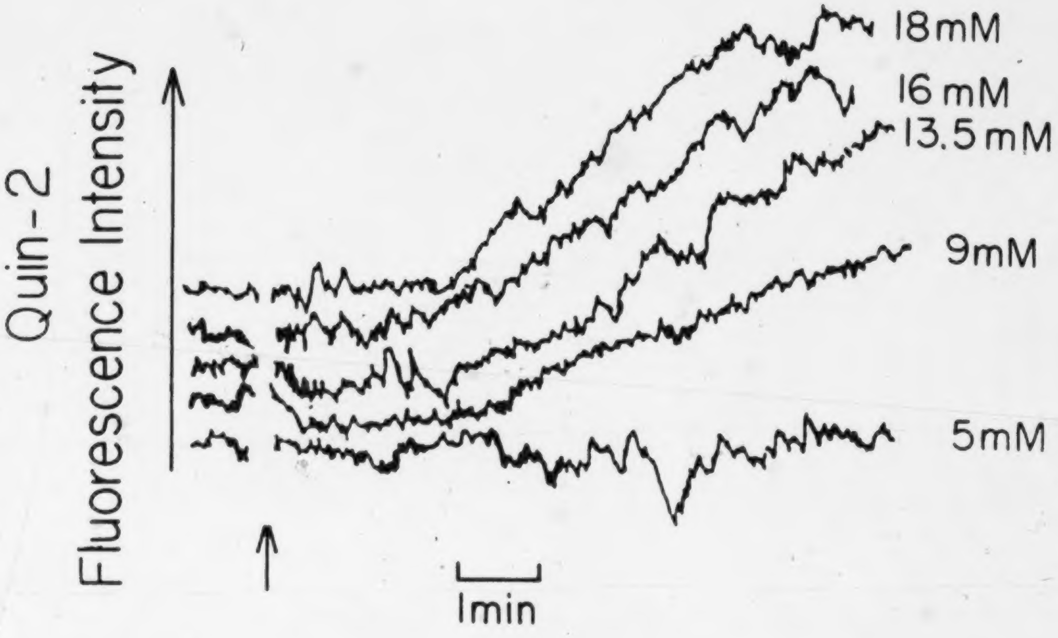


Figure 21: Relationship of Ca^{2+} mobilization to NaF concentration in a 2 X 10^6 cell/mL suspension of neutrophils. The arrow indicates the time of addition of stimulus. These results depict the tracings obtained from a single experiment which is representative of at least eight experiments.

concentration was characterized by a prolonged lag period of 4 to 10 min. Acceleration of the rate of calcium mobilization was observed to be dose-dependent up to an optimal concentration of 18 mM. At concentrations above 20 mM, the Ca^{2+} mobilization response appeared to decline due to autoinhibitory effects.

As reported previously (Molski et al., 1984), pertussis toxin inhibits the fmet-leu-phe-evoked rise in $(Ca^{2+})_i$ (Figure 22), as well as the associated superoxide production. Fmet-leu-phe-induced calcium mobilization differed from that elicited by fluoride in being of more rapid onset (maximal response within 15 sec), smaller magnitude (approximately 30-50%), and rapid termination (1-2 min). The time course of the fmet-leu-phe-stimulated rise in $(Ca^{2+})_i$ corresponds to that of the associated respiratory burst.

Results illustrated in Figure 22 show that fluoride-activated calcium mobilization was resistant to inhibition by pertussis toxin, suggesting that its mechanism of G-protein activation is unaffected by ADP-ribosylation of the protein. However, the fluoride-induced calcium response was largely abolished in cells pretreated with 2 mM EGTA for 10 min preceding the addition of NaF, indicating a strong dependence on calcium influx from the extracellular medium (Figure 23). However, as extracellular chelators also lead to a depletion of intracellular Ca^{2+} with time, these results should be interpreted with caution.

The simultaneous administration of fmet-leu-phe served to potentiate Ca^{2+} mobilization in response to fluoride (Figure 24), a phenomenon which has also been reported for the superoxide production response (Wong, 1983). Potentiation was manifested by a decrease in the

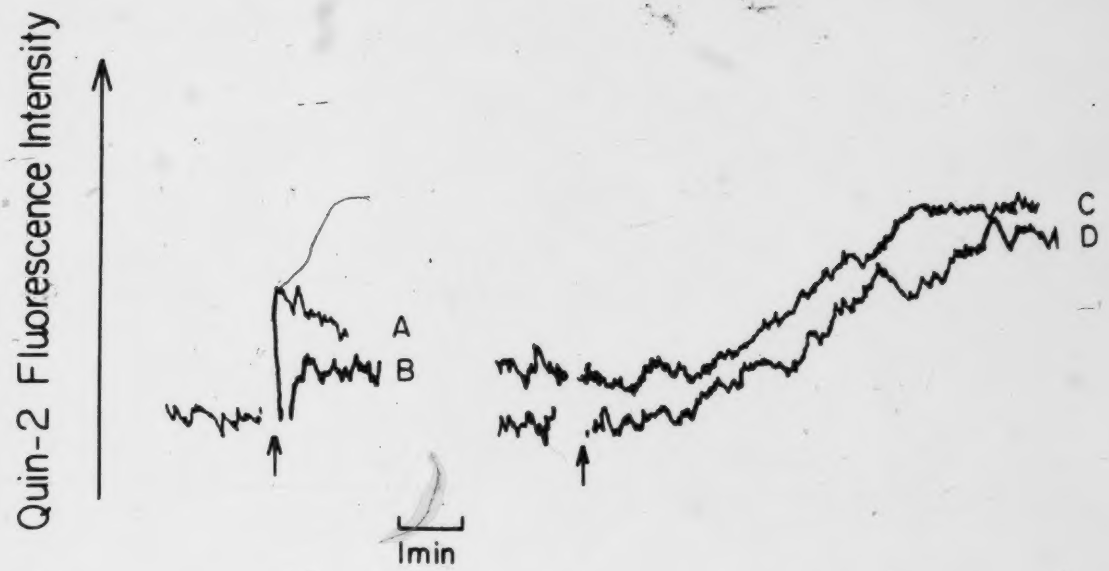


Figure 22: Differential pertussis toxin effects on fmet-leu-phe- and NaF-evoked calcium mobilization. Ca^{2+} mobilization by 1 μM fmet-leu-phe (A and B) and 18 mM NaF (C and D) was monitored in the presence (B and D) and absence (A and C) of a 60 minute preincubation with 1 μg/mL pertussis toxin. The results depict the tracings obtained from a single experiment which is representative of at least five experiments.

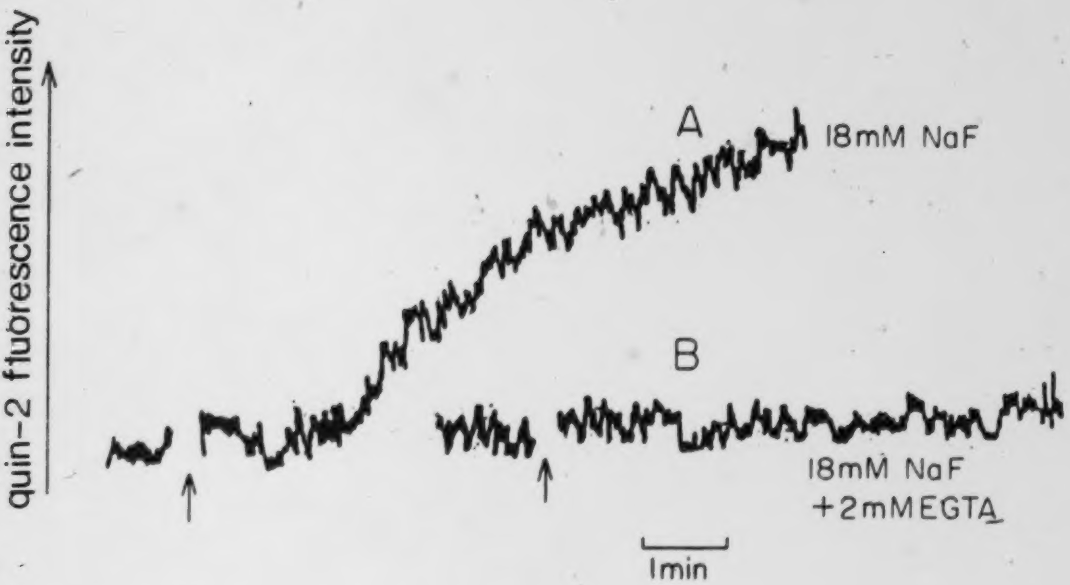


Figure 23: Ca^{2+} mobilization by 18 mM NaF in the presence and absence of 2 mM EGTA. The arrows indicate the time of addition of stimulus. The results depict the tracings obtained from a single experiment which is representative of at least five experiments.

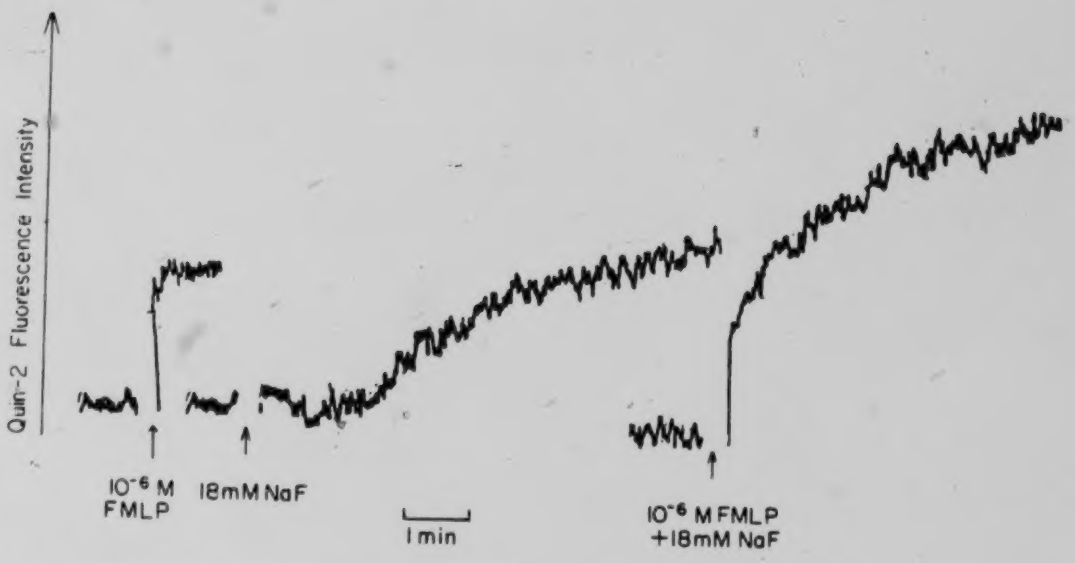


Figure 24: Interactions between fmet-leu-phe and NaF in the elevation of cytosolic calcium. Ca^{2+} mobilization responses were monitored for 1 μM fmet-leu-phe (FMLP) and 18 mM NaF alone and in combination. The results depict the tracings obtained from a single experiment which is representative of at least five experiments.

lag period preceding the fluoride component of the response (as determined by extrapolating the initial linear portion of the tracing to the baseline, represented, in this case, by the plateau of the fmet-leu-phe portion of the tracing), as well as acceleration of the initial rate (estimated from the slope of the tracing at the onset of the fluoride portion of the reaction).

F. FLUORIDE-INDUCED ACCUMULATION OF INOSITOL PHOSPHATE METABOLITES IN NEUTROPHILS

The aqueous samples obtained from acid extraction of control and fluoride-stimulated neutrophil suspensions were found to contain five peaks of $[^3H]$-activity when eluted through the anion exchange columns. Identification of the metabolites was performed by comparing the elution pattern of the unknown peaks with those of the commercially obtained standards for $[^3H]$inositol, $[^3H]InsP_1$, $[^3H]InsP_2$, and $[^3H]InsP_3$.

The first peak was not retained on the resin and appeared in the distilled water wash, in a manner identical to that of standard $[^3H]$inositol. The next four peaks, here designated A, B, C, and D, are shown in Figure 25. Peak A, previously identified as glycerophosphoinositol (Berridge et al. 1983), eluted with buffer 1 (5 mM sodium borate, 60 mM sodium formate). Peak B and the $InsP_1$ standard eluted in the same position with buffer 2 (0.1 M formic acid, 0.2 M ammonium formate), while peak C and the $InsP_2$ standard co-eluted with buffer 3 (0.1 M formic acid, 0.4 M ammonium formate). Peak D eluted with buffer 4 (0.1 M formic acid, 1 M ammonium formate), in a manner which coincided with the $InsP_3$ standard. Fluoride stimulation

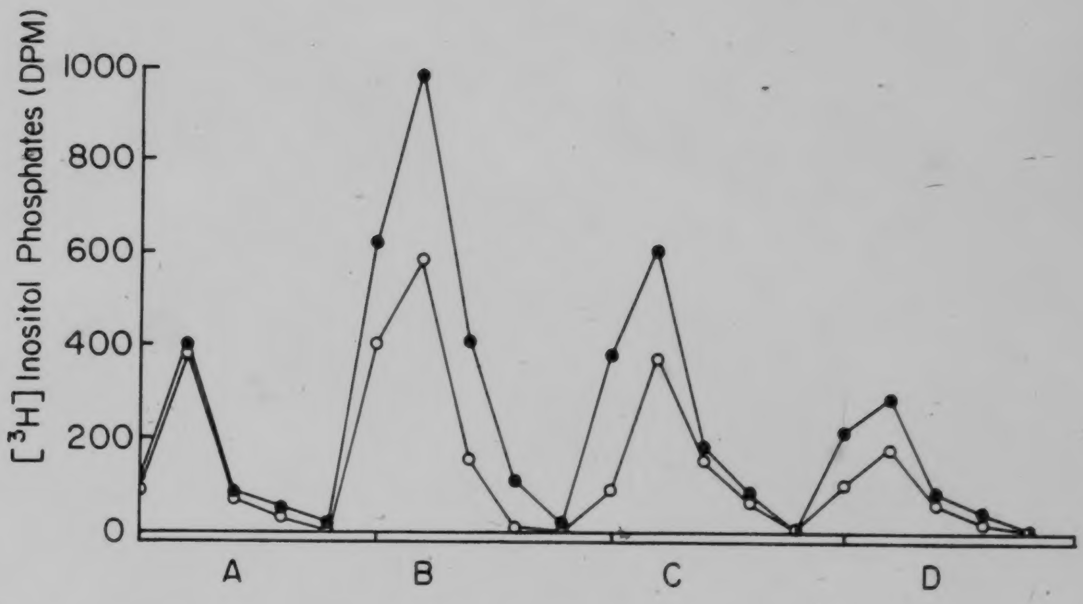


Figure 25: Elution profile of water-soluble extracts obtained from 20 X 10^6 cell/mL aliquots of [^{3}H]inositol-labelled neutrophils before (○) and after (●) a 20 min exposure to 18 mM NaF. Cell suspensions were pretreated for 10 min with 10 mM LiCl prior to removal of aliquots at the identicated time points. Peaks A through D were eluted from anion exchange resin of AG1 X8 200-400 mesh by a step gradient of the following elution buffers: A) 5 mM disodium tetraborate/60 mM sodium formate, B) 0.1 M formic acid/0.2 M ammonium formate, C) 0.1 M formic acid/0.4 M ammonium formate, and D) 0.1 M formic acid/1.0 M ammonium formate.

caused a large increase in peaks B and C, with a smaller increase in peak D and no change in peak A.

Owing to phosphatase-mediated inositol phosphate degradation, which tends to preclude the net accumulation of these metabolites during long term responses, 10 mM lithium chloride was added to reaction mixtures 10 minutes preceding the addition of stimulus to inhibit the breakdown of $InsP_1$ by $InsP_1$-phosphomonoesterase (Berridge et al., 1983; Drummond et al., 1984). Figure 26 shows the effect of lithium action on $InsP_1$ accumulation in response to fluoride. The time course of the generation of inositol 1-monophosphate ($InsP_1$), inositol 1,4-bisphosphate ($InsP_2$), and inositol 1,4,5-trisphosphate ($InsP_3$), following exposure of cells to 18 mM NaF, is shown in Figure 27. As is consistent with the superoxide production and calcium mobilization aspects of fluoride-induced neutrophil activation, phosphoinositide turnover was characterized by a sustained duration of action. Due to the site of action of this inhibitor, the major inositol phosphate detected was $InsP_1$. Because levels of $InsP_2$ and $InsP_3$ showed a fairly high degree of variability under conditions of lithium inhibition, subsequent studies focused on the $InsP_1$ formation.

The time course of fluoride-induced inositol phosphate production differed from that elicited by fmet-leu-phe (Figure 28). The latter agonist caused a rapid elevation of $InsP_2$ and $InsP_3$ levels within the first 20 sec of stimulation, followed by a slower increase in $InsP_1$ levels which became pronounced at 40 sec and remained elevated for approximately 2-3 min following addition of the stimulus. Conversely, the $InsP_3$ and $InsP_2$ peaks declined rapidly over the first 60-90 sec of

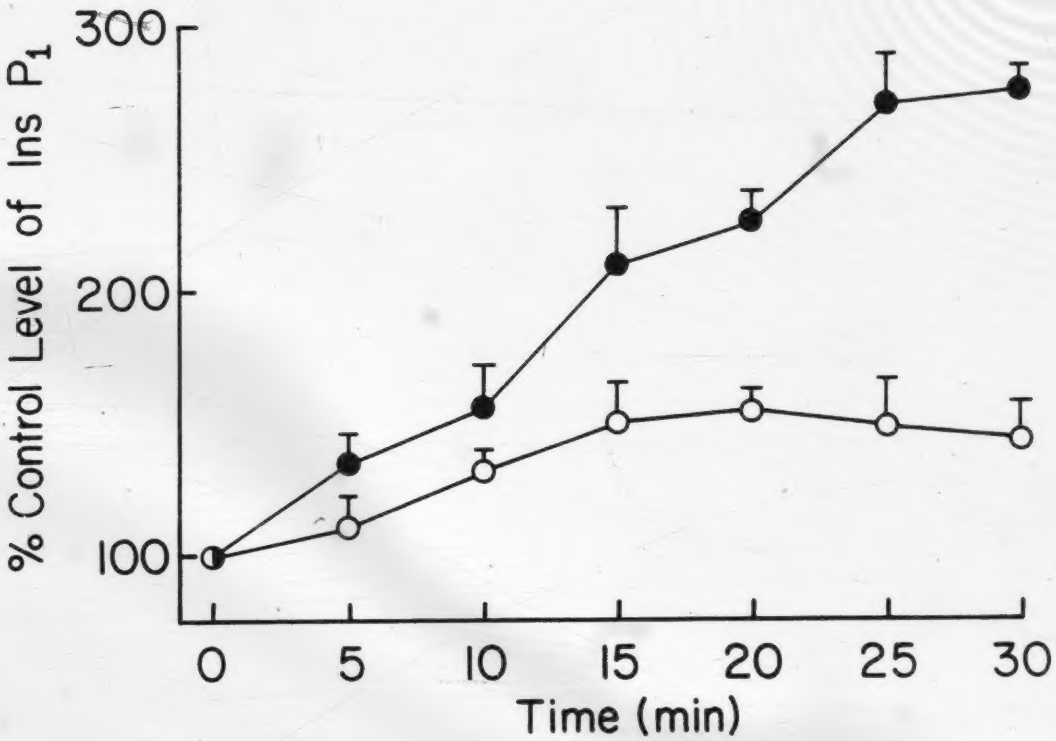
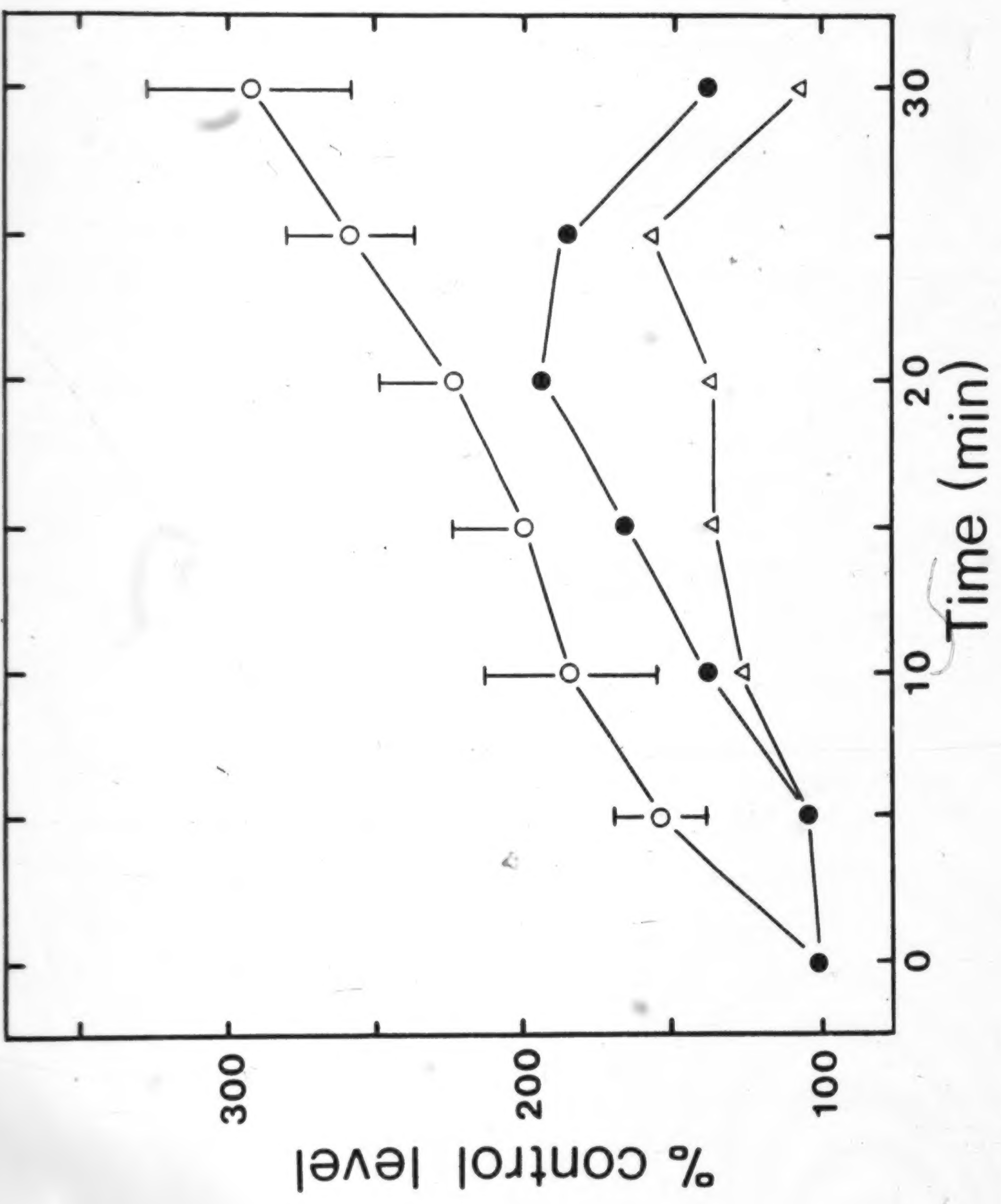


Figure 26: Effect of lithium on fluoride-activated $InsP_1$ production. Radiolabelled cell suspensions were preincubated for 10 min in the presence (●) or absence (○) of 10 mM LiCl prior to challenge with 18 mM NaF. Values shown represent the mean ± SEM for at least 3 determinations.

Figure 27: Time course of inositol phosphate accumulation in fluoride-activated neutrophils. Aliquots containing 2 X 10^7 cells, radiolabelled with [^{3}H]inositol and pretreated with 10 mM LiCl, were activated with 18 mM NaF for the time periods shown. Reaction periods were terminated by the addition of acid, whereupon the intracellular levels of the inositol phosphates were quantitated as described in Methods. Results are expressed as percent of control value (ie. levels in resting cells prior to NaF addition). The values for $InsP_1$ (○) represent the mean ± SEM for at least 6 independent experiments. The values for $InsP_2$ (●) and $InsP_3$ (△) represent the average of duplicate samples from a single experiment which is representative of at least 8 experiments. Control levels were 1,122.5 ± 450 dpm for $InsP_1$, 469 ± 126 dpm for $InsP_2$, and 277 ± 90 dpm for $InsP_3$ per 2 X 10^7 cell sample.


% control level
300
200
100
0
10
20
30
Time (min)

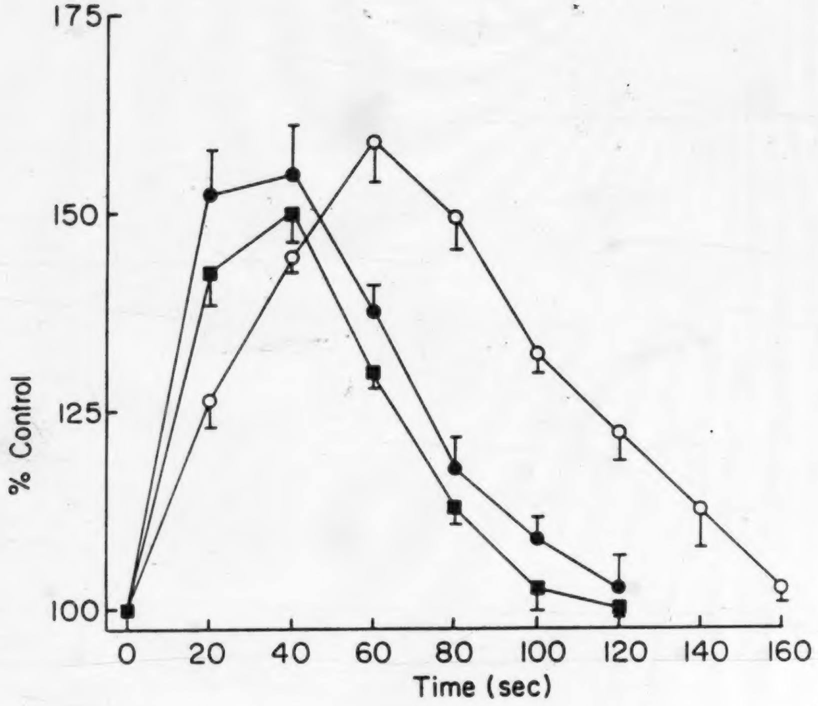


Figure 28: Time course of inositol phosphate production in neutrophils activated with 10^{-6} M fmet-leu-phe. The values for $InsP_1$ (○), $InsP_2$ (●), and $InsP_3$ (■) represent the mean ± SEM for at least 5 experiments. Control levels in the absence of lithium were in the range of 1,000 dpm for $InsP_1$, 450 dpm for $InsP_2$, and 300 dpm for $InsP_3$ per 2 X 10^7 cell sample.

stimulation. As lithium has no pronounced effect on short term responses (Bradford and Rubin, 1985a), it was excluded from the fmet-leu-phe time course study. However, in subsequent inhibitor studies, involving a comparison of fmet-leu-phe and fluoride responses, lithium was included in fmet-leu-phe incubation mixtures for the sake of consistency.

As reported previously (Becker et al., 1985; Bradford and Rubin, 1985b), pertussis toxin inhibited the fmet-leu-phe-evoked phosphoinositide turnover response, reinforcing the theory of G-protein involvement in the transduction pathway (Figure 29). Fmet-leu-phe-induced phosphoinositide turnover differed from that elicited by fluoride in being of more rapid onset (approx 15 sec) and termination (1-2 min). The time-course of fmet-leu-phe-stimulated $InsP_1$ accumulation correlated with that of the associated calcium mobilization and superoxide production. The comparative brevity of the chemotactic peptide-induced responses may be due to desensitization or down-regulation of the fmet-leu-phe receptor and/or inactivation of the G-protein, as a result of GTP hydrolysis or covalent modification.

Conversely, fluoride-activated $InsP_1$ accumulation was resistant to inhibition by pertussis toxin (Figure 29), suggesting that its mechanism of G-protein activation is unaffected by ADP-ribosylation of the protein. Fluoride-induced $InsP_1$ production also differed from that elicited by fmet-leu-phe in being resistant to inhibition by a 20 min preincubation with dibutyryl cAMP (Figure 30), a finding which corroborates our previous observation that fluoride-induced superoxide production is unaffected by adenylate cyclase agonists. However, a ten

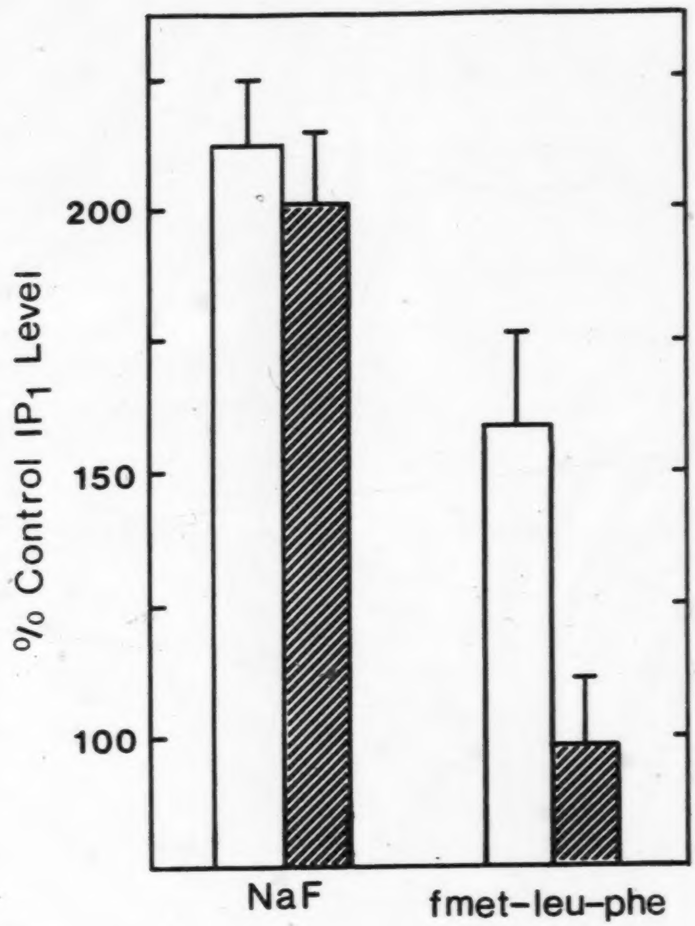


Figure 29: Effect of pertussis toxin on fluoride and fmet-leu-phe-activated InsP$_1$ production. Radiolabelled cell suspensions were preincubated for 90 min in the presence (crosshatched bars) or absence (open bars) of 1 µg/mL pertussis toxin prior to challenge with 1 µM fmet-leu-phe or 18 mM NaF for 60 sec and 20 min time intervals, respectively. Lithium chloride was added 10 min prior to fmet-leu-phe or NaF. Values represent the mean ± SEM of at least 5 determinations. The means for the two fluoride-treated groups were not significantly different, whereas those for the fmet-leu-phe-treated groups were ($p<0.05$).

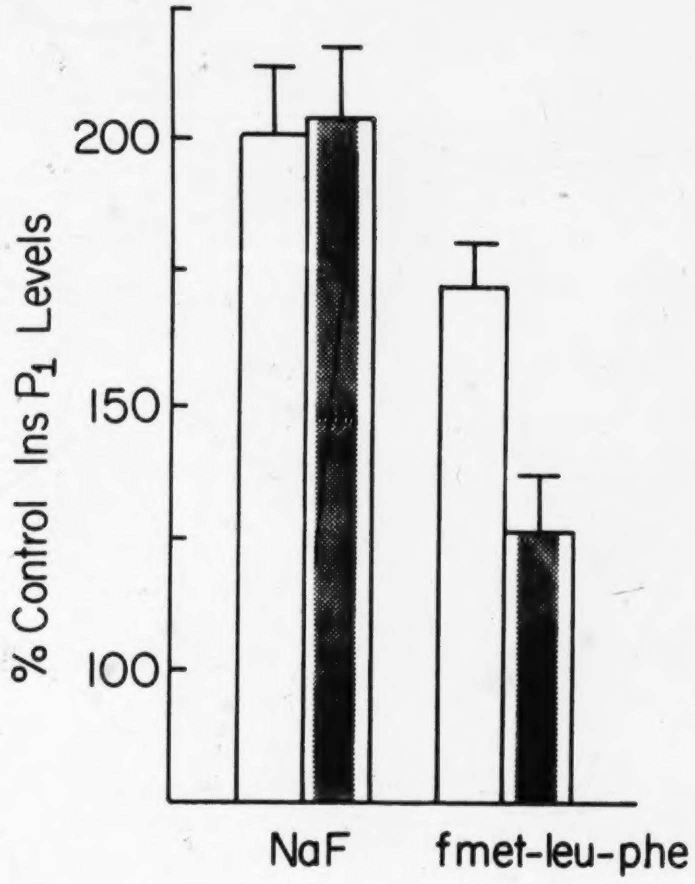


Figure 30: Effect of dibutyryl cAMP on fluoride- and fmet-leu-phe-activated $InsP_1$ production. Radiolabelled cell suspensions were preincubated for 30 min in the presence (shaded bars) or absence (open bars) of 1 mM dibutyryl cAMP prior to challenge with 1 μM fmet-leu-phe or 18 mM NaF. Values represent the mean ± SEM of at least 5 determinations. The means for the two fluoride-treated groups were not significantly different, whereas those for the fmet-leu-phe-treated groups were ($p<0.05$).

minute preincubation of cells with 1 µM PMA was found to inhibit subsequent phosphoinositide turnover in response to fluoride (Figure 31).

G. FLUORIDE ACTIVATION OF HUMAN NEUTROPHILS IS ASSOCIATED WITH THE TRANSLOCATION OF PROTEIN KINASE C ACTIVITY FROM THE CYTOSOLIC TO THE MEMBRANE COMPARTMENT

Inositol phosphate production in a stimulated cell system is associated with the concomitant generation of 1,2-diacylglycerol, a product linked with the activation of protein kinase C. In the resting cell, the majority of protein kinase C activity (5.8 ± 0.4 pmol ^{32}P/mg protein) was found to reside in the 100,000 X g supernatant, representative of the cytosolic fraction. A minor component (0.55 ± 0.04 pmol ^{32}P/mg protein) was localized in the 100,000 X g pellet, representative of the membrane fraction. Protein content in the samples was based of the mass of bovine serum albumin added, assuming that the amount of protein contributed by the samples was negligible. As the fractionation procedure used here involved no attempts to discriminate between the contribution of the various subcellular structures to the particulate fraction, the sum of the soluble and particulate fractions should be representative of the total cellular protein kinase C activity. Indeed, as reported by Wolfson et al. (1985), recovery of protein kinase C in the 100,000 X g soluble and particulate fractions, generally exceeds 100% of the total protein kinase C activity in whole cell sonicates.

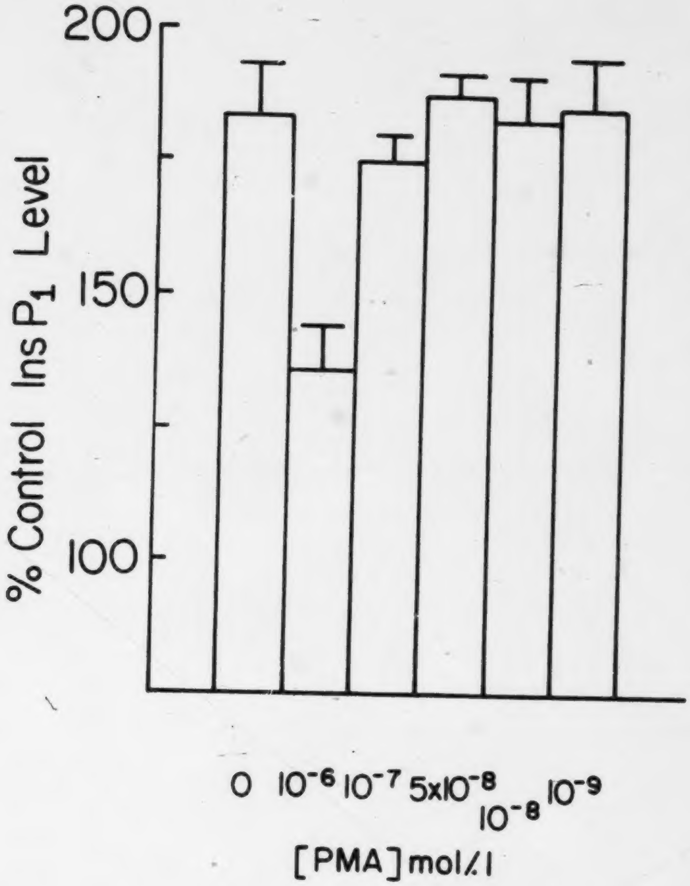


Figure 31: Effect of PMA on fluoride-activated $InsP_1$ production. Radiolabelled cell suspensions were preincubated for 10 min in the presence or absence of PMA prior to challenge with 18 mM NaF. Values represent the mean ± SEM of at least 5 determinations. A significant difference was detected only for cells treated with 1 μM PMA ($p<0.05$).

Activation of neutrophils with 18 mM NaF was characterized by the loss of protein kinase C activity from the cytosolic fraction and a concomitant increase in membrane-associated protein kinase C activity (Figure 32). This translocation event was preceded by a lag interval of 5 to 10 min and displayed a prolonged duration, a time course corresponding to that of the associated superoxide generation (Figure 29). Protein kinase C translocation appeared to be most pronounced in cells incubated with fluoride for 15 min, at which time a loss of approximately 85% of cytosolic protein kinase C was observed. After the 15 min time point, cytosolic kinase activity was observed to undergo a slow return to baseline levels over the next 10 min. Protein kinase C activity in the particulate fraction increased with a time course which correlated with that of the loss in cytosolic activity. An increase of approximately 60% over control was evident at the peak. A similar redistribution phenomenon was not detectable in cells stimulated with 1 μM fmet-leu-phe over 30 sec to 5 min time intervals.

Notably, the point of maximal translocation in response to fluoride, corresponded to that at which the rate of fluoride-induced superoxide production was maximal (15 to 20 min). Termination of the respiratory burst normally ocurred 30 to 60 min after the addition of fluoride to the cells, indicating that NADPH oxidase, the superoxide generating complex, continued to turn over for some time after complete recovery of cytosolic protein kinase C activity.

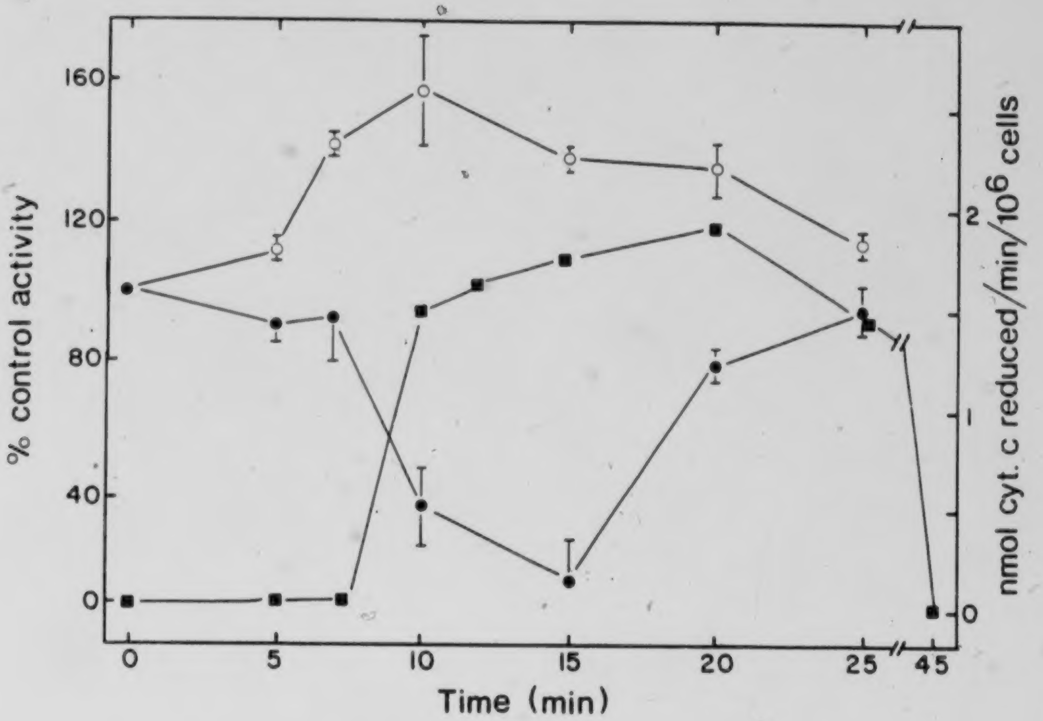


Figure 32: Time course of protein kinase C translocation and superoxide production rate in fluoride-activated neutrophils. Cell suspensions incubated with fluoride for fixed time intervals were assayed for protein kinase C activity in the cytosolic (●) and membrane (○) fractions, as described in Methods. The values shown represent the mean ± SEM of 6 experiments. The rate of superoxide production (■), as determined by the rate of reduction of cytochrome c, was determined at various time points on a continuous tracing curve. The values shown are based on a single experiment which is representative of at least 10 experiments.

CHAPTER IV

DISCUSSION

DISCUSSION

A. INTERACTIVE EFFECTS OF THE PHORBOL ESTER TUMOR PROMOTOR, PMA, AND THE CALCIUM IONOPHORE, A23187, ON SUPEROXIDE PRODUCTION IN HUMAN NEUTROPHILS

The major conclusion of this study was that protein kinase C activation by means of the phorbol ester, PMA, and cytosolic calcium elevation by the ionophore, A23187, interacted synergistically to elicit accelerated superoxide production and granular enzyme release responses in human neutrophils. These findings are in accordance with those of Robinson et al. (1984) in the guinea pig neutrophil system and White et al. (1984) in rabbit neutrophils.

Synergistic effects on the respiratory burst were observed in the form of a decreased lag interval preceding superoxide production, as well as an increase in superoxide generation rate. No change in total superoxide production was observed, however. Even at nanomolar concentrations, PMA continues to activate superoxide production until the response is exhausted. Cessation of the respiratory burst thus appears to be determined by limiting concentrations of some component of the superoxide generating system, possibly NADPH (Wong and Chew, 1985). The prolonged duration of PMA-induced responses is presumably due to the slow metabolism of this phorbol ester within the plasma membrane (Nishizuka, 1983).

Interactive effects on neutrophil degranulation were examined with respect to the release of lysozyme, an enzyme preferentially localized in the specific granules. Notably, PMA-induced degranulation appears to affect the specific, but not the azurophilic, granular pool (Goldstein et al., 1975; Wright et al., 1977). As reported by White et al. (1984), synergism between PMA and A23187 could be resolved only within the first 10 minutes following the addition of the stimuli, since, at maximally elevated calcium concentrations, A23187 elicits a massive degranulation response from the azurophilic, as well as the specific, granules which obscures resolution of the PMA component of the response. While 0.1 μM A23187 and 250 nM PMA evoked slow release responses within the first 5 min when applied alone, the combination of the two resulted in a rapid exocytotic response which exceeded the sum obtained with the two stimuli applied separately. Notably, the superoxide production and degranulation components of the inflammatory response exhibited differential sensitivity to the two stimuli, with degranulation being more potently evoked by the ionophore and superoxide production by the phorbol ester. These findings suggest that, in circumstances of receptor-mediated stimulation, the respiratory burst may be predominantly influenced by protein kinase C activation, while degranulation may be more significantly controlled by calcium mobilization. However, the recent findings that the protein kinase C inhibitors, polymyxin B and 1-(5-isoquinolinesulfonyl) piperazine inhibited PMA-induced degranulation (Naccache et al., 1985b) and superoxide production (Gerard et al., 1986), respectively, but not the chemoattractant-induced responses, has led to some questioning of the importance of protein kinase C in the transient responses elicited by fmet-leu-phe.

Previous studies have demonstrated that neutrophil activation by PMA leads to the phosphorylation of a 50,000 molecular weight protein, while elevated intracellular Ca^{2+} levels result in myosin light chain phosophorylation, probably through a calmodulin-dependent pathway. Both of these proteins are phosphorylated during neutrophil activation by the chemotactic peptide, fmet-leu-phe (Fechheimer and Zigmond, 1983; White et al., 1984).

Robinson et al. (1984) suggest that the synergism between A23187 and PMA may represent partial activation of protein kinase C by sub-optimal PMA concentrations, such that the kinase exhibits a calcium affinity which, although still somewhat greater than ambient intracellular concentrations, is particularly sensitive to intracellular calcium elevation by A23187. However, it is equally attractive to suggest that A23187 and PMA each stimulate independent transduction processes which interact synergistically in the activation of NADPH oxidase and exocytotic processes. A23187 probably contributes through a calmodulin-dependent process activated by the ionophore-induced influx of extracellular Ca^{2+}. PMA presumably intercalates into the plasma membrane where it substitutes for diacylglycerol, one of the products of phosphoinositide turnover, in the activation of the Ca^{2+}- and phospholipid-dependent protein kinase, protein kinase C (Nishizuka, 1983). Since physiological stimuli, such as fmet-leu-phe, are known to induce both phosphoinositide breakdown (Cockcroft, 1982) and an increase in cytosolic Ca^{2+} (White et al., 1983), it seems plausible that both protein kinase C- and calmodulin-dependent mechanisms are at work in naturally occurring inflammatory responses, producing efficient cellular responses to small amounts of the second messenger substances, $InsP_3$ and diacylglycerol. The synergistic interaction of phorbol esters and calcium ionophores has been extended to a multitude of other experimental systems, including histamine release from rat mast cells (Katami et al., 1984), mitogen stimulation of peripheral lymphocyte proliferation (Mastro and Smith, 1983), steroidogenesis in bovine adrenocortical cells (Culty et al., 1984), insulin release from rat pancreatic islets (Zawalich et al., 1983), and catecholamine release from bovine adrenal medullary cells (Knight and Baker, 1983).

When A23187 concentrations exceeded 1 µM, inhibition of superoxide production rate and total superoxide production was readily observed in PMA-stimulated neutrophils. The ionophore appeared not to be cytotoxic by the criterion of lactic dehydrogenase release. Furthermore, a maximal degranulation response under these circumstances suggested a specific toxicity for the superoxide production component of the response. Inhibition may have resulted from either a direct effect upon NADPH oxidase, itself, or interference with some event in the transduction processes preceding activation of the oxidase complex. As neither synergism nor inhibition were observed in Ca^{2+}-free media, these effects did not appear to be attributable to non-specific disruption of the membrane by the ionophore. Perhaps when cytosolic Ca^{2+} concentrations exceed a critical level, certain calcium-sensitive steps in the activation pathway become desensitized. Although A23187 concentrations above 0.5 µM are known to result in nonspecific activation of phospholipase C, phospholipase A_2, and protein kinase C (Nishizuka, 1984), it is unclear how any of these events would have a negative effect on superoxide radical production.

If both calcium and protein kinase C are required to achieve the necessary phosphorylation pattern for a maximal respiratory burst, why do high PMA concentrations evoke maximal superoxide production alone? A possible explanation for this phenomenon resides in the non-specific effects of phorbol esters which become evident at concentrations of 0.1 µM and more. For example, high doses of phorbol ester have been reported to promote the fusion (Kaibuchi et al., 1983) and perturbation (Yamanishi et al., 1983) of cell membranes, effects which may modify the localization and activity of various molecular components involved in superoxide generation. Furthermore, much information is accumulating to suggest that PMA is not entirely specific for the calcium- and phospholipid-dependent protein kinase. Calcium-dependent hydrophobic interaction chromatography (Malviya et al., 1986) has enabled the resolution of a calcium-independent, but phospholipid-dependent and PMA/diacylglycerol-activated, kinase from the calcium- and phospholipid-dependent protein kinase C component of bovine brain extract. Activation of protein kinase L, as this novel kinase system has been termed, may account for the ability of high PMA concentrations to elicit maximal responses in the absence of calcium. Incidentally, it is presently unclear whether protein kinase L represents a single enzyme or a family of kinases.

B. PHORBOL ESTER EFFECTS ON PROSTAGLANDIN- AND CHEMOATTRACTANT-STIMULATED CYCLIC AMP PRODUCTION

In the neutrophil, elevation of cytosolic cAMP levels through use of the lipid-permeable cAMP analogue, dibutyryl cAMP, or by means of adenylate cyclase activation, through use of prostaglandin E_1, cholera toxin, or forskolin, caused inhibition of chemoattractant-induced superoxide production. Such findings are in confirmation of the work of Simchowitz et al. (1980) and Wong and Freund (1983). Conversely, the phorbol ester-induced respiratory burst was exempt from these inhibitory effects. In view of numerous reports of phorbol ester-mediated inhibition of hormonally stimulated adenylate cyclase, PMA's effects on prostaglandin- and chemoattractant-induced cAMP accumulation were investigated.

Preincubation of human neutrophils with 0.1 μM PMA was not found to inhibit cyclic AMP elevation in response to either PGE_1 or the chemoattractant, fmet-leu-phe. In fact, phorbol ester pretreatment actually served to enhance the subsequent cyclic AMP elevation in response to these agents, without affecting the basal cAMP levels in unstimulated cells. Such a finding contrasts with the situation in avian erythrocytes (Kelleher et al., 1984), mouse Leydig cells (Mukhopadhyay et al., 1984), and rat hepatocytes (Heyworth et al., 1984) in which phorbol ester pretreatment leads to a reduction of hormonally-induced cyclic AMP production.

These results are, however, in accordance with studies using membrane preparations from PMA-pretreated platelets (Jakobs et al., 1985) which show that, whereas cyclase stimulation in the presence of

PGE_1 and GTP was greatly enhanced by the phorbol ester, hormonal inhibition was severely impaired. This phenomenon was associated with increased phosphorylation of a 41K protein, upon exposure of human platelet membranes to exogenous, partially purified protein kinase C (Katada et al., 1985). The 41K substrate has been suggested to be the activated form of N_i on the basis of studies using purified N_i and its subunits which indicate that the α_i monomer was a good phosphorylation substrate, while the $\alpha\beta\gamma$ N_i oligomer was not. Significantly, addition of the purified β subunit of N_i to platelet membranes specifically inhibited 41K phosphorylation. This interpretation was reinforced by a study using cyc^- S49 cells, which lack functional N_s, but not N_i, G-regulatory proteins. While phorbol ester treatment did not stimulate forskolin-mediated cAMP elevation in this cell line, it did impair N_i-induced inhibition of the cyclase by somatostatin.

Although β-adrenergic agonists and prostaglandins of the E series are known to operate predominantly through activation of N_s, a certain amount of N_i-mediated cyclase inhibition is also observed (Murayama and Ui, 1983; Asano et al., 1985). Murayama and Ui (1983) have speculated that this phenomenon may represent an N_s-mediated activation of N_i. Alternatively, Asano et al. (1985) have suggested the possibility of a degree of receptor "cross-talk," whereby β-adrenergic agonists may have secondary interactions with N_i, in addition to the primary receptor-N_s interactions. Regardless of the mechanism, it is appealing to interpret these results as being indicative of an inherent control mechanism whereby excessive stimulatory input can be diminished. In my intact cell studies, it appears plausible that the observed enhancement of

PGE_1-activated cAMP production in phorbol ester-treated cells may represent the relief of a degree of inhibitory tone which normally imposes some constraint on agonist-induced cyclase activation. However, since it is unclear whether cAMP production in response to fmet-leu-phe is a result of eicosanoid generation (Hopkins et al., 1984), cAMP-phosphodiesterase inhibition (Verghese et al., 1985b), or perhaps some as yet unsuspected mechanism, we cannot be certain whether phorbol ester enhancement of the chemoattractant-induced cAMP response is attributable to N_i inactivation or to influences at some other site.

The fact that fairly high phorbol ester concentrations are necessary to observe enhancement of cAMP accumulation suggests that perhaps this effect of protein kinase C activation may be of minor physiological relevance. However, considering the susceptibily of chemoattractant-induced cell activation to inhibition by cAMP, it is conceivable that, under circumstances of a high level of cell activation, protein kinase C's effects on the adenylate cyclase system may promote physiological antagonism by prostaglandins and other cyclase activators, thus serving as a form of protection against over response. While enhancement of fmet-leu-phe-induced cAMP elevation is of lesser magnitude, it, too, may represent a negative regulatory mechanism in situations of excessive protein kinase C activation.

C. ACTIVATION OF LYSOZYME RELEASE BY GUANINE NUCLEOTIDE ANALOGUES

The calcium-dependent release of lysozyme occurring in permeabilized and resealed cells loaded with the non-hydrolyzable guanine nucleotide analogues, Gpp(NH)p and GTPγ-S, provides evidence for the involvement of one or more guanine nucleotide-binding proteins in the stimulus-secretion coupling mechanism. These effects were not mimicked or influenced by any of the adenine nucleotides tested nor by GDP. Effects of GTP were erratic, probably due to the rapid metabolism of this nucleotide by intracellular phosphatases.

Presumably, the non-hydrolyzable guanine nucleotide analogues would promote a persistently activated state of N_n, the G-protein intermediating between chemoattractant receptors and the phosphoinositide-specific phosphodiesterase. The resulting stimulation of phosphodiesteratic phosphoinositide metabolism would be manifested by calcium mobilization and protein kinase C activation. By analogy with the situation in intact cells, the resultant calcium mobilization and protein kinase C activation would elicit Ca^{2+}-dependent and Ca^{2+}-independent secretory signals which would, through distinct phosphorylation events, contribute to the degranulation response observed.

The fact that Ca^{2+}-independent degranulation was not observed may be reflective of excessive depletion of intracellular calcium concentrations during permeabilization in the calcium-free EGTA-containing buffer. While, protein kinase C appears not to be a calcium-regulated enzyme, its activation by diglyceride is dependent on the presence of approximately 10^{-7} M calcium in the cytosol (Kishimoto et al., 1980). Likewise, it is probable that guanine nucleotide-activated phospholipase C activity might be suppressed under conditions in which cytosolic calcium levels were severely depleted. Furthermore, as protein kinase C, in its resting state, exists mainly as either a soluble or loosely membrane bound enzyme (Kraft and Anderson, 1983), it is possible that, during a detergent permeabilization procedure, an appreciable loss of the enzyme may occur through plasma membrane lesions, as is evidently the case for lactic dehydrogenase.

The putative localization of guanine nucleotide analogue-mediated cell activation to a G-protein transducing unit communicating with the phosphoinositide-specific phosphodiesterase was later substantiated by Cockcroft and Gomperts (1985) who observed that GTPγ-S can activate the polyphosphoinositide phosphodiesterase in human neutrophil membranes at the 10^{-7} M calcium concentration characteristic of the intracellular milieu (millimolar calcium concentrations are required for the Ca^{2+}-dependent activation of the enzyme). Phosphodiesterase activation was monitored by the loss in PtdIns(4,5)P_2, as well as the increase in $InsP_2$ and $InsP_3$. Further evidence that G-protein involvement is responsible for phosphodiesterase activation was provided by the demonstration that, in mast cells loaded with GTPγ-S together with 10 to 100 μM neomycin, a drug which prevents phosphoinositide hydrolysis, the guanine nucleotide-induced degranulation response upon subsequent addition of calcium was abolished.

Smith et al. (1986), using plasma membranes obtained from human neutrophils, discovered that receptor occupation by fmet-leu-phe in the presence of GTP was associated with a reduction in the calcium requirement for phospholipase C activation from millimolar concentrations to the physiological 10^{-7} M range, possibly due to an increase in the affinity of the phosphodiesterase for calcium. This response could be prevented by pretreatment of the membranes with pertussis toxin.

Although the use of membrane preparations imposes certain constraints, such as the inability to observe functional responses to biochemical changes, it has the advantage of unequivocally dissociating the effect of agents on plasma membrane structures from possible sites of action on intracellular regulatory proteins. As discussed by Barrowman et al. (1986a), this is probably not the case when dealing with guanine nucleotide-loaded permeabilized cells. In a study involving the effect of calcium and guanine nucleotides on β-glucuronidase release from rabbit neutrophils permeabilized with Sendai virus, they found that GTPγ-S can induce granular secretion and activation of the phosphodiesterase in the absence of calcium (ie. in cells loaded with chelator substances during permeabilization), an observation which they feel may be indicative of another GTP-sensitive site involved at a distal point in the exocytotic process. As GTP sensitivity has been reported for both membrane fusion (Paiement, 1984) and microtubule assembly processes (Sandoval and Weber, 1980), either of these functions may be possible targets for GTP involvement in secretory events.

In my studies, Ca^{2+}-insensitive release could potentially be occurring during the actual loading procedure. However, when lysozyme release was monitored during the interval of exposure to saponin, no detectable increase in lysozyme release could be observed for cells permeabilized in the presence, as opposed to the absence, of guanine nucleotide analogues. Furthermore, it is important to note that lysozyme release, which was addressed in our studies, and β-glucuronidase release, which was examined by Barrowman et al. (1986a), represent the exocytosis of two separate granular pools which are known to be subject to different regulatory mechanisms. β-Glucuronidase is localized in the azurophilic granules, while lysozyme, though common to both classes of granules, is found predominantly in the specific. Whereas lysozyme release can be elicited by either the calcium or protein kinase C pathways independently or synergistically by both (White et al., 1984), PMA is reported to inhibit the Ca^{2+}-activated release of β-glucuronidase (Barrowman et al., 1986b). Moreover, although PMA was found to elicit β-glucuronidase release in permeabilized cells, this effect of the phorbol ester was not characteristic of intact neutrophils (Goldstein et al., 1975; Wright et al., 1977)

In view of some of the discrepancies between results obtained in intact and permeabilized neutrophils, it is necessary to mention that cell permeation techniques are not without inherent disadvantages which render it difficult to extrapolate all of the results observed in these studies to normal physiological situations. One major issue is that the state of equilibration attained during loading of exogenous solutes is difficult to quantify. The administered concentration of loading solute will rarely reflect that achieved in the intracellular compartment. Moreover, the inevitable loss of lactic dehydrogenase and other cytosolic factors makes the permeabilized cell a potentially artifactual system. Altered plasma membrane properties may be inferred from the cells' tendency to clump following the permeabilization procedure. Furthermore, different neutrophil preparations showed a fairly wide range of variability in their ability to tolerate exposure to saponin, often rendering it necessary to alter the duration of the actual permeabilization procedure between individual experiments. These problems led us to seek an alternative approach to G-protein activation which would be applicable to studies using intact, unpermeabilized cells.

D. FLUORIDE ACTIVATION OF THE NEUTROPHIL RESPIRATORY BURST

Exposure of human neutrophils to millimolar concentrations of sodium fluoride was observed to result in the release of superoxide into the extracellular medium. This response was characterized by a prolonged lag period which may reflect the time interval required for the ion to reach its site of action in the membrane, suggestive of an integral membrane protein as the receptor site. As the cell has no specialized transport system for fluoride, its passage across the membrane presumably represents a passive process. The usual threshold concentration was 9 to 10 mM with 16 to 18 mM proving to be the optimal concentrations. At high fluoride concentrations, autoinhibitory effects resulted in the premature termination of the response. As this autoinhibitory phenomenon is even more pronounced in isotonic sucrose (Curnutte et al., 1979), it does not appear to be a reflection of increased hypertonicity. Furthermore, controls performed in modified

HBSS, in which the NaCl concentration was reduced in correspondence to the amount of NaF added, did not show any appreciable alteration in the kinetics of fluoride-induced superoxide production.

The fluoride-induced superoxide production response was totally unaffected by preincubation procedures with pertussis toxin and dibutyryl cAMP which almost completely abolished the fmet-leu-phe-induced respiratory burst, suggesting that fluoride's mechanism of action is resistant to the major negative modulator processes, perhaps partially accounting for its sustained duration with respect to the chemoattractant-induced responses.

Fluoride activation of the respiratory burst was, however, a calcium-sensitive phenomenon, as observed by the decrease in the rate and total amount of superoxide generated in the presence of Ca^{2+}-free media supplemented with EGTA. Conversely, PMA-induced superoxide production was unaffected under the same circumstances, indicating that the absence of Ca^{2+} must interfere with the transduction of the fluoride stimulus, rather than with the actual superoxide generation process. These findings prompted us to perform a qualitative examination of intracellular Ca^{2+} changes during neutrophil activation by fluoride.

E. CALCIUM MOBILIZATION IN FLUORIDE-ACTIVATED NEUTROPHILS

The results of this study show that fluoride induced an increase in intracellular free calcium levels, as monitored by the fluorescent calcium probe, Quin 2 (Strnad and Wong, 1985b). The calcium mobilization response paralleled that of fluoride-activated superoxide production, in having a prolonged lag period and sustained duration of action. This finding is consistent with the hypothesis that fluoride elicits a respiratory burst by means of the persistent activation of a G-protein. The extended duration of the fluoride-evoked calcium response, as compared with that elicited by fmet-leu-phe, is consistent with the fact that fluoride is known to induce dissociation of G-proteins into their constituent subunits, in a manner which is not readily reversible (Katada et al., 1984a).

Fluoride-induced activation of the putative N_n was unaffected by preincubation of cells with pertussis toxin under conditions which largely abolished the fmet-leu-phe response. This finding is in accordance with the observation that pertussis toxin fails to interfere with N_i-mediated inhibition of agonist-stimulated adenylate cyclase, when N_i activation is achieved through the use of fluoride (Katada et al., 1984a,b). It should be noted that Bokoch and Gilman (1984) did observe pertussis toxin inhibition of fluoride-stimulated arachidonic acid release. However, the dose-response profiles for the calcium and superoxide responses differed from that for arachidonic acid release, with optimal concentrations being 18 mM for the former, and 50 mM for the latter. Since marked inhibition of arachidonate release by pertussis toxin occurred only at fluoride concentrations of 20 mM and above, concentrations at which calcium mobilization and superoxide production were already subject to autoinhibition, it is difficult to correlate these two phenomena.

Fluoride-induced calcium mobilization appeared to be largely dependent upon calcium influx from the extracellular medium, as indicated by the reduction of this response in EGTA-containing medium. It thus appears that the fluoride-induced elevation of cytosolic calcium levels is not wholly dependent on Ins(1,4,5)P_3-induced calcium release from the endoplasmic reticulum and probably also involves an element of calcium influx at the plasma membrane level. However, at present, the molecular mechanisms involved in the regulation of calcium movement across the plasma membrane remain unresolved. Moreover, as EGTA treatment is associated with a certain amount of intracellular, as well as extracellular, Ca^{2+} depletion, the respective contribution of intracellular and extracellular calcium supplies to the calcium mobilization response is difficult to estimate on the basis of chelator effects.

The potentiating effects of fmet-leu-phe on fluoride-induced calcium mobilization may be accounted for by an acceleration of fluoride's ability to gain access to the guanine nucleotide-binding site on the G-protein, where it is postulated to exert its pharmacological effects (Bigay et al., 1985), as will be discussed in more detail below. According to Rodbell's "Disaggregation Theory of Hormone Action" (Rodbell, 1980), G-proteins, in their inactive state, exist with their guanine nucleotide-binding sites in a predominantly closed state. Opening of this site would presumably occur at a slow rate. Interaction of the G-proteins with agonist-activated receptors favours an increase in the rate of "opening and closing" of the guanine nucleotide-binding site, simultaneously promoting access of fluoride to its active site. Conceivable, in the absence of the fmet-leu-phe "priming" effect, the amount of fluoride-activated G-protein does not reach threshold amounts until the observed lag interval has elapsed.

F. PHOSPHOINOSITIDE TURNOVER IN FLUORIDE-ACTIVATED NEUTROPHILS

By monitoring the accumulation of inositol phosphates, the water-soluble products of phosphoinositide turnover, we demonstrated that the generation of these metabolites occurred with a time course adequately accounting for the associated calcium mobilization response (Strnad et al., 1986). Fluoride-induced $InsP_1$ accumulation was unaffected by either pertussis toxin or dibutyryl cAMP, but was inhibited by high concentrations of PMA.

The failure of $InsP_2$ and $InsP_3$ to be highly elevated throughout the response is partially attributable to their rapid breakdown to $InsP_1$ in lithium-treated cells (Berridge et al., 1982). However, by analogy with other systems, it is probable that, in the neutrophil, $PtdIns(4,5)P_2$ degradation and the concomitant generation of $Ins(1,4,5)P_3$ occur only during the initial stage of stimulation, with PtdIns breakdown and Ins(1)P generation predominating in the latter phases of the response (Majerus et al., 1985). It is also likely that the $InsP_3$ which is detected at these later time points is not $Ins(1,4,5)P_3$, but $Ins(1,3,4)P_3$ (Irvine et al., 1985; Burgess et al., 1985). The fact that intracellular Ca^{2+} levels remain elevated throughout the response, despite the fact that $Ins(1,4,5)P_3$ levels are probably not consistently elevated, suggests the involvement of some factor modulating Ca^{2+} fluxes at the level of the plasma membrane. Such a mediator would account for the extracellular Ca^{2+}-dependency of fluoride-evoked superoxide production and calcium mobilization. Although there has been some speculation that $Ins(1,3,4,5)P_4$, produced by the phosphorylation of $Ins(1,4,5)P_3$, may serve to regulate Ca^{2+} channels in the plasma membrane (Hansen et al., 1986), this hypothesis yet to be substantiated. Furthermore, in view of recent reports concerning a putative G-protein mediating calcium release at the level of the endoplasmic reticulum (Gill et al., 1986), it is conceivable that fluoride effects on calcium elevation may be attributable to actions at sites on both the plasma membrane and the microsomes.

Unfortunately, the isotopic techniques currently available preclude the possibility of actually quantitating the levels of inositol phosphates produced in stimulated cells. In addition to the problem of pool equilibration, a situation never approximated in the comparatively short incubation procedure suitable for neutrophils, the existence of two isomers of inositol trisphosphate, $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$, and inositol phosphate, $Ins(1)P_1$ and $Ins(4)P_1$, which co-elute on the standard anion exchange procedure (Michell, 1986), further complicate attempts to estimate the actual concentration of these metabolites achieved during activation. As the cyclic derivatives of the inositol phosphates do not survive acid extraction procedures, there is no potential for detecting these metabolites by the technique reported here. Moreover, the tetrakisphosphate, $Ins(1,3,4,5)P_4$, a product which is gaining increasing recognition, cannot be adequately resolved by passive anion exchange chromatography. Resolution of these species awaits the development of high pressure liquid chromatographic techniques in this laboratory.

G. PROTEIN KINASE C REDISTRIBUTION IN FLUORIDE-ACTIVATED NEUTROPHILS

The translocation of protein kinase C activity from the cytosolic to the membrane fraction of fluoride-activated cells was found to occur with a time course which correlated with the associated cellular responses (Strnad et al., 1986). Protein kinase C activation can be viewed as an indirect measure of diacylglycerol formation resulting from phosphoinositide degradation.

While protein kinase C activity was localized primarily in the cytosolic fraction of resting neutrophils, fluoride activation was associated with a dramatic loss of this soluble kinase activity which was accompanied by increased kinase activity in the particulate fraction, presumably indicative of the enzyme forming a state of intimate association with the plasma membrane upon its conversion to the activated form (Kraft and Anderson, 1983).

Incidentally, the apparent localization of protein kinase C in the cytosolic fraction of unstimulated cells is probably an artifact of the EDTA-containing buffer used during cell disruption. Divalent ion chelation would tend to extract those peripheral membrane proteins bound to the membrane in a calcium-dependent manner. Helfman et al. (1983) reported that when cells were homogenized in the absence of calcium chelators, protein kinase C expressed a weak affinity for the particulate fraction, typical of a peripheral protein.

Interestingly, the majority of chemoattractants which interact with cell surface receptors appear to be minimally dependent on protein kinase C activation. In our studies, fmet-leu-phe did not elicit detectable protein kinase C translocation, an observation corroborating those of Nishihira et al. (1986) with respect to fmet-leu-phe, platelet-activating factor, and leukotriene B_4. These investigators have suggested that the readily reversible state of cell activation elicited by these chemoattractants may bypass protein kinase C activation, in spite of a considerable degree of phosphoinositide metabolism. An alternative interpretation, would be that protein kinase C activation through receptor-mediated events involves a much smaller proportion of the total kinase pool than that available for activation by fluoride or PMA. PMA would have, as potential targets, the entire protein kinase C pool, while fluoride would affect that proportion of the pool which would be in proximity to the diacylglycerol generated by the N_n-activated phosphodiesterase. However, chemoattractant activation would influence only that subpopulation of N_n recruited by the receptor type in question, thus resulting in a much smaller quantity of diacylglycerol generated. It is thus conceivable that, while chemoattractant-induced kinase translocation may occur, the magnitude of this response defies the resolution capacities of the assay system employed.

The fact that the loss of protein kinase C activity from the cytosol was not associated with a quantitative recovery of activity in the membrane fraction of fluoride-activated cells is a common feature of this type of assay (Wolfson et al., 1985). It appears that detergent solubilization is either insufficient to extract all of the membrane-associated kinase activity or else results in a partial inactivation of kinase function. Indeed, the unusual resistancy of neutrophil protein kinase C to detergent extraction has led Wolfson et al. (1985) to propose that the protein kinase C complex may be associated with the cytoskeleton. This is particularly interesting in view of the finding of Nishihira et al. (1986) that, while fmet-leu-phe alone did not result in detectable protein kinase C translocation, challenge of cells with this chemoattractant in the presence of the actin-interacting drug, cytochalasin B, allowed the resolution of a certain amount of kinase redistribution. Furthermore, it is known that, once bound to the membrane, protein kinase C is subject to inactivation by proteolytic degradation (Tapley and Murray, 1984) and phosphorylation (Kikkawa et al., 1982; Rozengurt et al., 1983), situations which, despite the use of protease inhibitors in the assay buffer, may partially account for the lack of quantitative recovery of activity in the membrane fraction.

H. USE OF FLUORIDE ION AS A PROBE FOR THE GUANINE NUCLEOTIDE-BINDING PROTEIN INVOLVED IN PHOSPHOINOSITIDE TURNOVER

The ability of fluoride to induce superoxide production, Ca^{2+} mobilization, and protein kinase C translocation, in association with phosphoinositide turnover, implicates an early stage in the transduction process as the site of action of this inflammatory stimulus. Fluoride-evoked effects were characterized by a slow onset and sustained duration. This time course contrasts with that of chemoattractants, such as fmet-leu-phe, which exploit a traditional cell surface receptor mechanism that invariably displays a lag period of 15 sec or less and rapid desensitization. The delayed and prolonged response to fluoride thus seems more likely to represent the persistent activation of an integral membrane protein involved in the signal transmission process. Such a site of action is consistent with our proposal that the G-protein transducing unit, interposed between the receptor and the phosphodiesterase, is the probable site of action of fluoride as an inflammatory stimulus. Although our present data cannot preclude the possibility that fluoride is acting directly on the phosphodiesterase, we regard this possibility as unlikely, considering fluoride's well-established role as an activator of guanine nucleotide-binding proteins in the adenylate cyclase system. However, in view of the recent finding (Gill et al., 1986) that calcium release at the level of the endoplasmic reticulum may involve a guanine nucleotide-sensitive regulatory protein, we cannot ignore the possibility that fluoride-evoked calcium mobilization may be occurring at two sites: one involving G-protein activation of phospholipase C and another involving activation of a G-protein-mediated signal transduction process in the endoplasmic reticulum.

In support of these interpretations, a study conducted simultaneously and independently by Blackmore et al. (1985) has documented the effect of NaF on the pertussis toxin-insensitive guanine nucleotide-binding protein involved in the phosphoinositide-dependent cell activation mechanism of the hepatocyte. In this cell system, millimolar concentrations of NaF were found to elicit activation of phosphorylase, inactivation of glycogen synthase, calcium efflux, elevation of cytosolic calcium concentrations, and a decrease in

$PtdIns(4,5)_2$ levels with a concomitant elevation of $InsP_3$ and diacylglycerol. These changes occurred with a more rapid time course than their counterparts in the neutrophil, the lag period being only 1 min and the duration 2 to 5 min.

Taylor et al. (1986) subsequently demonstrated $InsP_3$ accumulation in electrically permeabilized cells from rat parotid gland upon exposure to NaF, as well as in the presence of GTPγS. These investigators mentioned the fact that fluoride's ability to inhibit the $Ins(1,4,5)P_3$ 5'-phosphatase (Storey et al., 1984) which degrades $Ins(1,4,5)P_3$ to $Ins(1,4)P_2$ may contribute somewhat to the $InsP_3$ component of fluoride-induced phosphoinositide turnover, although this effect could not explain the increase in other phosphoinositide metabolites noted in the hepatocyte and the neutrophil.

Our findings (Strnad and Wong, 1985) concerning fluoride-induced calcium mobilization in neutrophils led Poll et al. (1986) to readdress the effect of NaF on human platelets, in which fluoride has a recognized ability to evoke dense granule release (Murer, 1968; Murer et al., 1981). They found that fluoride-induced degranulation in platelets was accompanied by elevated cytosolic calcium concentrations and the generation of thromboxane B_2. Lag periods in platelets tended to be shorter than in neutrophils; but, dose-effects were similar. They also confirmed the dependence of fluoride-induced calcium mobilization on extracellular calcium, reporting inhibition of this response, as well as degranulation and thromboxane B_2 generation, in cells exposed to 2 mM EGTA 1 min before fluoride stimulation. Their finding of PMA inhibition of fluoride-induced intracellular calcium elevation corresponds well with our finding that high concentrations of PMA suppressed the phosphoinositide turnover response elicited by fluoride.

I. MECHANISM OF ACTION OF FLUORIDE AS A G-PROTEIN ACTIVATOR

Fluoride's action on G-proteins has a demonstrated requirement for trace amounts of aluminum (Sternweis and Gilman, 1982). Indeed, the quantities of aluminum present in solutions stored in common glassware are quite adequate for this purpose (Gabler et al., 1983). Efficiency appears to be maximal at those concentrations of sodium fluoride at which the fluoroaluminate complex, AlF_4^-, is favoured (Goldstein, 1964; Matwiyoff and Wageman, 1970).

Bigay et al. (1985) advanced an attractive proposal for the mechanism of action of fluoride as a G-protein activator. Using the light-activated rhodopsin-transducin-cGMP phosphodiesterase system (Stryer et al., 1981) as their model, they developed reconstituted systems in which the purified transducin α subunit (T_α-GDP) and the partially purified phosphodiesterase were restored to retinal outer segment (ROS) membranes which had been deprived of all protein components except rhodopsin. The addition of NaF and $AlCl_3$ to this reconstituted system conferred upon T_α-GDP the ability to activate the phosphodiesterase in the absence of a light stimulus, in a manner comparable to that of T_α-GTPγS.

In an extension of this study, using purified bovine ROS membranes containing a native complement of transducin and phosphodiesterase, they demonstrated that the addition of NaF and $AlCl_3$ in the dark induced dissociation of T_α-GDP from $T_{\beta\gamma}$. Notably, the presence of GDP in the binding site appeared to be a strict requirement. Fluoride was ineffective in cases where the site was empty, a condition achieved by removing all guanine nucleotides in the presence of a strong illumination stimulus--circumstances in which activated rhodopsin leads to the displacement of bound GDP from T_α, but not its subsequent replacement. Significantly, fluoride activation was restored by the addition of GDP. However occupation of the guanine nucleotide site by GDPγS prevented fluoride action, indicating that the large S atom somehow encroaches on the AlF_4^- binding domain.

Dose-activation curves suggested that the stoichiometry of this effect involved the binding of one AlF_4^- per T_α unit with a binding constant of 1 μM or higher. Apparently, fluoride, in the millimolar range, was capable of inducing conformational changes in the GDP-bound α-subunit which were equivalent to those of the GTP-bound state. This was reinforced by the finding that AlF_4^--activated T_α-GDP exhibited the same proteolytic sensitivity characteristics as T_α-GTPγS (Stein et al., 1984; 1985). These findings led Bigay et al. (1985) to propose that AlF_4^- acts by mimicking the gamma phosphate of GTP.

This mimicry is believed to be accounted for by certain structural similarities between AlF_4^- and PO_4^{-3}. Notably, both are tetrahedral. The central aluminum and phosphorus atoms are the same size. Fluoride and oxygen both have Van der Waal's radii of 1.35 Angstroms. The P-O bond length is 1.55 to 1.60 or Angstroms, while the Al-F bond is 1.65-1.70 Angstroms. The extended duration of fluoride-induced activation can be attributed to the high electronegativity of fluoride which would result in stronger binding than that possible for the gamma phosphate of GTP which is readily removed by the intrinsic GTPase activity of the α-subunit (Bigay et al., 1985).

J. REGULATION OF CHEMOATTRACTANT- AND FLUORIDE-INDUCED PHOSPHOINOSITIDE TURNOVER

Both protein kinase A and protein kinase C activation, as well as pertussis toxin-mediated ADP-ribosylation, have been found to exert negative effects on the activation of the phosphoinositide turnover pathway in the neutrophil (Della Bianca et al., 1986a; Della Bianca et al., 1986b; Bradford and Rubin, 1985; Smith et al., 1985; Volpi et al., 1985). As regulation by these inhibitors seems to be related to various functional alterations in the GTP-binding or GTPase properties of N_n, it was interesting to examine the effects of these changes on the fluoride activation process.

Unlike chemoattractant-induced cellular responses, superoxide production, phosphoinositide turnover, and calcium mobilization in response to fluoride ion were unaffected by pertussis toxin pretreatment. As ADP-ribosylation by pertussis toxin inhibits GTPγS binding to N_n, but not GTPγS-induced activation of the protein, it would appear that this toxin brings about inhibition by interfering with the GDP-GTP exchange process (Smith and Snyderman, 1986). Notably, ADP-ribosylation of N_n by pertussis toxin is inhibited by GTPγS, suggesting a preference for the inactive undissociated G-protein as a substrate. As fluoride's mechanism of action is a GTP-independent process targetted at the GDP-bound form of N_n, it is not surprising that ADP-ribosylation does not affect fluoride activation of the protein.

Cyclic AMP-mediated inhibition of neutrophil responses is exploited by a number of physiological antagonists such as prostaglandins of the E series (Lad et al., 1985b; Takenawa et al., 1986). Likewise, the transient chemoattractant-induced elevation of cAMP levels may contribute to limiting the extent of cell activation (Verghese et al., 1985b). Notably, the activation of cellular responses by phorbol ester tumor promotors and calcium ionophores is unaffected by cyclic AMP, while chemoattractant responses are inhibited (Fujita et al., 1984; Della Bianca et al., 1986). Conflicting reports state that fmet-leu-phe binding is either unaffected (Snyderman et al., 1986) or inhibited (Holian et al., 1986) by cyclic AMP pretreatment. The finding that cAMP inhibits chemoattractant-induced phosphoinositide turnover has led to the suggestion that either N_n or phospholipase C may be a potential target of cAMP-mediated inhibition. Our finding that fluoride-induced phosphoinositide turnover was unaffected by preincubation of cells with dibutyryl cAMP, under conditions which almost completely abolished fmet-leu-phe-induced phosphoinositide turnover, indicate that phospholipase C is unlikely to be the target of cAMP inhibition. This would implicate either or both the fmet-leu-phe receptor and the G-protein as the sites of protein kinase A phosphorylation. If the latter possibility is correct, it would imply that fluoride activation of the G-protein must somehow be resistant to the inhibitory effects of cAMP. This would suggest that the inhibitory mechanism is dependent on the presence of a hydrolyzable phosphate group in the γ position of the active site. Inhibition might take the form of a decreased rate of guanine nucleotide exchange, a form of inhibition which would not affect the action of fluoride whose target is the GDP-bound G-protein. Alternatively, it might involve an increase in the terminating GTPase activity in which case, again, a hydrolyzable γ phosphate would be necessary for the expression of the inhibitory effects.

High concentrations of PMA have been found to inhibit calcium mobilization (Lagast et al., 1984; Schell-Frederick, 1984), degranulation (Naccache et al., 1985a) and phosphoinositide turnover in response to fmet-leu-phe (Della Bianca et al., 1986b). In contrast to pertussis toxin, PMA appears to inhibit GTPγS-induced phosphoinositide turnover, but not fmet-leu-phe-induced GTPγS binding (Smith and Snyderman, 1986). Interesting, Katada et al. (1985) indicated that PMA preferentially phosphorylated dissociated α_i, but not oligomeric N_i. Conceivably, the same preference in substrates might be anticipated for N_n. Matsumoto et al. (1986) noted that phorbol esters inhibited both the basal and fmet-leu-phe-activated high-affinity GTPase activity of neutrophil homogenates, as well as increasing the pertussis toxin-induced ADP-ribosylation of a 41K protein. Notably the latter effect was not observed in homogenates, suggesting that inhibition may be exerted at more than one level of G-protein function.

Although PMA was found to attentuate fluoride-induced phosphoinositide turnover, the high concentration required for this inhibition exceeded those at which a substantial degree of cell activation and protein kinase C translocation occur. This probably reflects that fact that fluoride activation of N_n involves, as potential targets, the entire N_n pool, whereas chemoattractant-induced activation of this G-protein would influence only those N_n oligomers which are associated with the cell surface receptor in question. Presumably, saturating concentrations of phorbol ester are necessary to result in the level of protein kinase C activation necessary to reduce a substantial component of the fluoride activatable pool of N_n. Nevertheless, at these levels of phorbol ester, the possiblity of non-specific effects cannot be ignored. It is, therefore, appropriate to suggest the possiblity that protein kinase L involvement or phorbol ester-induced changes in membrane properties may contribute to the inhibition of phosphoinositide turnover observed under these conditions.

The implications of fluoride ion as a probe for the guanine nucleotide-binding protein in neutrophils are multifold. Although it is regrettable that no pharmacological activator has yet been identified with the ability to selectively distinguish between various forms of the G-proteins, fluoride provides a useful tool with which to stimulate neutrophil activation at the level of N_n without resorting to the potentially artifactual membrane preparations or cell permeabilization procedures necessary for G-protein activation with GTP or its analogues.

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